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THE NEUROENDOCRINE CONTROL OF MATERNAL BEHAVIOUR IN BIRDS

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Lay Summary of Thesis

Maternal behaviour encompasses the care that a mother from a species that shows maternal care provides for her offspring with the goal of ensuring their immediate survival and success in later life. While maternal care in mammals has been extensively studied, not enough is known about the mechanisms that control it in birds. Maternal behaviour is accompanied by changes in specific hormones which are produced in the brain and are responsible for controlling the behaviour. In birds, these hormones include mesotocin and vasotocin which control social behaviours and are involved in the regulation of stress, as well as gonadotropin inhibitory hormone (GnIH) which inhibits sexual behaviours. Some sex steroid hormones (hormones which promote sexual behaviours and include testosterone, progesterone and estradiol) are known to interact with these avian neuropeptides or their mammalian equivalents. Sex steroid levels change during the reproductive cycle.

The aim of this PhD thesis was to examine more closely the changes in a number of brain hormones throughout the reproductive cycle in birds, their possible roles in maternal behaviour and related behaviours, and the relationships between them.

The experiments presented in this thesis demonstrated that testosterone and estradiol can influence the expression of vasotocin and mesotocin in the chicken brain, showing a relationship between sex steroids and these brain peptides in specific brain areas involved in maternal and other social behaviours.

The results from this project also showed that in the paraventricular nucleus of the hypothalamus (PVN) – a part of the brain strongly involved in maternal care – hens who were rearing their chicks expressed more mesotocin than hens laying eggs, suggesting a role for mesotocin in chick rearing. Both mesotocin and vasotocin expression decreased during incubation in the

lateral bed nucleus of the stria terminalis (BnSTl) – a brain area involved in social behaviours and stress. This could be explained by hens having less social interaction while incubating and possibly being less responsive to stress during that period, as high levels of stress are associated with high vasotocin. However, further studies are necessary to test these possibilities as stress and social interaction were not measured during the experiments presented here.

It was found that cells producing the GnIH protein were significantly higher in number in the brains of hens that were incubating their eggs compared to hens laying eggs. This result agrees with previous data and suggests that GnIH may be necessary to decrease sexual behaviours in favour of incubation.

The expression of the hormone prolactin, which is known to promote incubation, was measured in the pituitary gland throughout the reproductive cycle. The expression in the same gland for dopamine receptor 2 (D2), which is known to inhibit prolactin, was also measured. No differences in expression were found for either between hens at different stages of their reproductive cycle (laying eggs, incubating the eggs or rearing chicks).

No differences were found between the concentrations of the monoamine neurotransmitters adrenaline, noradrenaline, dopamine and serotonin or their precursors/metabolites in the raphe nucleus of the brain between the different stages of the reproductive cycle. Evidence from previous studies suggested that these monoamines may be involved in maternal behaviour and stress, but the results of the present project did not support this hypothesis. However, these results should be interpreted with caution due to issues with the accuracy of the procedure and the quality of the tissue used.

To determine how birds responded to chicks compared to adult individuals and identify the areas involved in interacting with chicks, the differences in behaviour were measured along with how activated the brains of Japanese

quail became when introduced to chicks vs adults of the same species. The activation of certain brain areas can be measured by measuring levels of marker proteins expressed in activated neurones. It was found that quail spent significantly more time in close proximity to new chicks than to a new adult and some areas in their brains involved in maternal and other social behaviours showed higher neuronal activation in the group introduced to chicks. However, in one area – the raphe nucleus – involved in both maternal behaviour and stress, activation was lower in the presence of chicks than in the presence of a new adult. It is possible that female quail found chicks to be a less stressful stimulus than a novel adult. However, as stress was not measured in the experiments in this project, further studies are necessary to test this.

The work presented in this thesis sheds more light on the hormones and mechanisms which control maternal care in birds and contributes to the areas of neuroscience, physiology, developmental biology and endocrinology.

Declaration

I declare that this thesis has been composed entirely by the candidate, Yana Aleksandrova. This work has not previously been submitted for a Doctor of Philosophy, a degree or any professional qualification. All sources of information and all contributions have been acknowledged. I have done all the work with the following exceptions:

- Tissue collection was carried out on different occasions by myself, Prof Simone Meddle, Valerie Bishop and Georgia Longmoor.
- Steroid injections were carried out on different occasions by myself and Prof Simone Meddle.
- Liquid chromatography – mass spectrometry sample preparation was carried out by myself and Dr Andrew Gill and equipment was operated by Dr Andrew Gill.
- Sex determination PCR, testing of PCR primers and qPCR was performed by myself and Angus Reid.
- Cryosectioning of brains was performed by myself, Elisa Nicklas, Cheryl Rae, Jessica Buchard and Jessica Stirling.
- Plasmid probe against GnIH for DIG *in situ* hybridisation was designed and provided by Dr Yasuko Tobari.

2018 Yana Aleksandrova

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Abstract

Maternal behaviour in humans and animals promotes the survival and future success of offspring. It is mainly controlled by the brain behaviour network in the hypothalamus. The behavioural and physiological changes which occur when an animal 'becomes maternal' are significant and include the cessation of reproductive behaviours and often a modulation of the stress response, energy balance and aggression. While the mammalian maternal brain has been extensively studied, much less is known in birds. This PhD project aimed to investigate the peptides and neuroendocrine pathways which govern the onset and maintenance of avian maternal care.

In the mammalian brain, maternal behaviour and other social behaviours are regulated by the nonapeptides oxytocin and vasopressin, which are also involved in the stress response and water balance. The oxytocin and vasopressin orthologues in birds and reptiles, called mesotocin and vasotocin, are very similar in structure and perform similar roles. However, very few studies have focussed on their involvement in maternal care. In the experiments presented in this thesis, it was found that mesotocin mRNA expression was higher in the paraventricular nucleus (PVN) of hens rearing chicks than in laying hens as early as the first day of rearing, suggesting the involvement of mesotocin in rearing in the chicken. In a subdivision of the lateral bed nucleus of the stria terminalis (BnSTl), mesotocin mRNA expression was lower during incubation, compared to laying and rearing, while vasotocin mRNA expression was lower in both incubating hens and hens on the first day of rearing, compared to layers. This differed from the expected results, as data from previous studies suggested that mesotocin in the medial BnST (BnSTm) of birds promoted maternal behaviour. Even though, to the knowledge of the author, the lateral sector of this nucleus had not been separately examined in this context prior to this project, results were expected to be similar, as the medial and lateral BnST are closely related and often examined together. It was speculated that the changes in the BnSTl which were observed could be connected to a possible decrease

in social interactions in chickens during incubation. Incubating hens very rarely leave the nest and actively discourage others from approaching it. This could theoretically lead to fewer interactions with their conspecifics. However, no formal measurement of sociality and social interactions was performed during this project and, since the chickens in the relevant experiment were housed in pairs with both birds often entering incubation at roughly the same time, it would have been difficult to judge what their behaviour would have been if the rest of the flock had been present. A measurement of social interaction should be included in further experiments investigating the significance of nonapeptide changes in the BnSTl during incubation.

With regards to vasotocin, it was speculated that the lower mRNA expression during incubation in the BnSTl could be related to an attenuated stress response in maternal birds. This suggestion was based on previous studies in other avian and mammalian species which have shown that parental animals can have lower responsiveness to stress. However, the stress response was not measured in this study and future work is necessary to determine whether it is indeed attenuated in the chicken under these conditions.

In order for maternal behaviour to take place, sexual behaviours need to be inhibited. Gonadal steroids, which control sexual behaviours, have been shown to interact with nonapeptides in the brains of mammals and birds, but these interactions are complex, context-dependent and not fully understood. The results of this project showed that acute treatment with certain sex steroids after a period of priming was capable of significantly increasing the expression of mesotocin or vasotocin mRNA in specific areas of the brain behaviour network. In both the PVN and BnSTl, testosterone but not its metabolite estradiol increased mesotocin expression, suggesting that the action of testosterone on mesotocin was direct. Vasotocin expression was increased only in the BnSTl by both testosterone and estradiol, suggesting that the action of testosterone on vasotocin in this brain area was likely achieved through estradiol, following the aromatisation of testosterone.

Gonadotropin inhibitory hormone (GnIH) is produced in the hypothalamus and has a strong inhibitory effect on the reproductive axis. GnIH shows significant changes throughout the reproductive cycle in the brains of both mammals and birds. After examining the changes in GnIH throughout the hen reproductive cycle, it was found that hens on the fourteenth day of incubation had a significantly higher number of GnIH-immunoreactive cells in the PVN compared to laying hens. These results were in agreement with previous findings and suggested that GnIH may be involved in downregulating reproductive behaviours during incubation.

The hormone prolactin is involved in maternal care in mammals and birds and it is important in incubation and rearing. It is known to be controlled by the dopaminergic system through the D1 dopamine receptor (D1, D1R), which promotes, and the D2 dopamine receptor (D2, D2R), which inhibits its expression. In this project, quantitative polymerase chain reaction (qPCR) was used to measure prolactin and D2 mRNA in the pituitary glands of hens throughout the reproductive cycle, from laying eggs through to the first day of rearing chicks. No changes in mRNA expression for either prolactin or D2 were observed, suggesting that changes in their mRNA expression in the pituitary gland are not crucial for the display of incubation and rearing. It is possible that other mechanisms contribute strongly to the increase in plasma prolactin during incubation.

Apart from controlling prolactin, dopamine is also involved in maternal and other social behaviours, such as sexual behaviour, social approach, social attachment, social dominance and aggression. Other monoamines, including serotonin (5-hydroxytryptamine, 5-HT), noradrenaline and adrenaline have also been implicated in social interactions, including aggression and maternal behaviour. All of these monoamines are present in the raphe nucleus but, to the author’s knowledge, the changes in their concentrations in this brain area throughout the reproductive cycle from egg-laying through to chick-rearing had not been characterised in birds prior to this project. Liquid chromatography - mass spectrometry (LC-MS) was used to examine the concentrations of monoamines in the raphe nucleus of the female chicken throughout the reproductive cycle and test the hypothesis that they

might be involved in maternal care in this species. No differences were found between groups for any of the examined monoamines, which included adrenaline, noradrenaline, dopamine, 5-HT, the dopamine precursor dihydroxyphenylacetic acid (DOPAC), the 5-HT precursor tryptophan and tryptophan's metabolite hydroxyanthranilic acid.

These results do not provide any evidence that monoamines in the raphe nucleus play a role in incubation or rearing in the chicken. However, as only monoamine content rather than release was measured, a role mediated by differences in release cannot be excluded. In addition, limitations of the experimental procedure mean that results from this experiment should be interpreted with caution.

Many animals display negative responses to the young of their species when not in a maternal state but habituation to young individuals can often alter these behaviours and produce a maternal response. The display of maternal care induces c-fos (an immediate early gene, marker of neuronal activation) expression in brain areas controlling the behaviour. The effects of social stimulation with chicks vs adults were tested in Japanese quail. The change of species from chicken was necessary due to unforeseen issues at the Roslin Institute Poultry Unit which led to the loss of the existing colony of maternal chickens. Unfortunately, despite many efforts, the colony could not be replaced in time for the aforementioned experiment to be conducted on the original species of choice. Japanese quail were the best replacement species the author had access to. While they rarely incubate eggs in captivity, they do display rearing behaviour, in addition to also being precocial, like the chicken, and having physiology and brain organisation similar to chickens.

It was found that adult female Japanese quail habituated to chicks for 6 days spent significantly more time in close proximity to novel chicks compared to novel adult individuals and stimulation with chicks caused greater c-fos expression than stimulation with a novel adult in a brain area related to maternal and other social behaviours (the PVN) and, surprisingly, an area related to sexual behaviour (the nucleus of the commisurae pallii, nCPa).

What type of cells were activated remains unclear and sexual behaviour was not tested for. In contrast, c-fos expression was lower in the group presented with chicks in the raphe nucleus – an area known to be involved in maternal behaviour, as well as stress. It can be speculated that, after habituation, chicks may have induced the beginning of maternal behaviour in females (and therefore higher activation in PVN neurones). They may have also presented a less stressful stimulus than a new adult (inducing less activation in the raphe nucleus). However, full maternal behaviour was not observed within the scope of this experiment and stress was not measured. Therefore, further studies are necessary to examine the significance of these brain areas in interactions with chicks, including determining what type of neurones were being activated.

This thesis presents an overview of the changes throughout the reproductive cycle in some major hormones of the brain behaviour network and examines their possible roles in maternal behaviour through specific brain nuclei. It provides evidence for the involvement of the mesotocin/vasotocin and GnIH systems in the control of maternal care in birds and sheds more light on the complex relationship between sex steroids and the brain nonapeptides. The findings presented here contribute to the areas of neuroscience, physiology, developmental biology and endocrinology.

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5-HT – 5-hydroxytryptamine, serotonin
6-OHDA – 6-hydroxydopamine
ABC – avidin-biotin complex
ADH – adenohypophysis
AM – nucleus anterior medialis hypothalamic
ANOVA – analysis of variance
anti-DIG-AP – anti-dioxygenine alkaline phosphatase
BLASTn – Basic Local Alignment Search Tool for nucleotides
BnSTl – Lateral bed nucleus of the stria terminalis
BnSTm – medial bed nucleus of the stria terminalis
CA – anterior commisure
cAMP – cyclic adenosine monophosphate
Cb – cerebellum
CORT – corticosterone
CRH – corticotropin releasing hormone
D1, D1R – dopamine receptor D1
D2, D2R – dopamine receptor D2
DAB – 3,3'-diaminobenzidine
DARPP-32 – dopamine- and cAMP-regulated neuronal phosphoprotein
ddH_2O – double-distilled water
DES – diethylstilbestrol

dH_2O – deionised water
DHT – dihydrotestosterone
DIG – digoxigenin
DLAmc – nucleus dorsolateralis anterior thalami, pars lateralis
DMSO – dimethyl sulphoxide
DNA – deoxyribonucleic acid
DOPA – 3,4-dihidroxyphenilalanine
DOPAC – dihydroxyphenilacetic acid
DPX – distyrene, plasticiser, xylene
EDTA – ethylenediaminetetraacetic acid
EGR-1 – early growth response protein 1
ER-α – estrogen receptor alpha
ER-β – estrogen receptor beta
FPL – lateral forebrain bundle
FPM – medial forebrain bundle
FSH – follicle stimulating hormone
GCt – substantia grisea centrlis, periaqueductal gray
gDNA – genomic deoxyribonucleic acid
GnIH – gonadotropin inhibitpry hormone
GnRH – gonadotropin releasing hormone
GRs – glucocorticoid receptors
H_2O – water
H_2O_2 – di-hydrogen peroxide
HA – hyperstriatum accessorium
Hp – hippocampus
HRP – horseradish peroxidase
HV – hyperstriatum ventrale
ICo – nucleus intercollicularis
ICV – intracerebroventricular
IgG – immunoglobulin G
IH – nucleus inferioris hypothalamic
IHC – immunohistochemistry
INF – infundibular nucleus

IP – intraperitoneal

ISH – *in situ* hybridisation

IV – intravenous

KCl – potassium chloride

KH_2PO_4 – potassium di-hydrogen orthophosphate

KO – knockout

LC-MS – liquid chromatography-mass spectrometry

LH – luteinising hormone

LHy – lateral hypothalamic area

LS – lateral septum

MCH – melanin-concentrating hormone

ME – median eminence

MeA – medial amygdala

MOS – medial olfactory stria

MPOA – medial preoptic area

mRNA – messenger ribonucleic acid

MS – medial septum

Na_2HPO_4 – di-sodium hydrogen orthophosphate

NaCl – Sodium chloride

NaPPI – sodium inorganic pyrophosphate

NBT-BCIP – nitroblue tetrazolium chloride - 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt

nCPa – nucleus of the commissurae pallii

NGS – normal goat serum

NH – neurohypophysis

nI – nucleus intermedialis

NPY – neuropeptide Y

NTP – nucleotide triphosphate

OC – optic chiasma

OM – tractus occipitomesencephalicus

OTR – oxytocin receptor

OVLT – organum vasculosum of the lamina terminalis

PBS – phosphate buffered saline

PBS-T – phosphate buffered saline - triton
PCR – polymerase chain reaction
PET – positron emission tomography
PHN – nucleus periventricularis hypothalami
POM – medial preoptic area (birds), nucleus preopticus medialis
POMC – proopiomelanocortin
POP – nucleus preopticus periventricularis
PRLR – prolactin receptor
PV – portal vessels
PVN – paraventricular nucleus
PVO – organum paraventriculare
RNA – ribonucleic acid
ROT – nucleus rotundus
RT – room temperature
SCNm – nucleus suprachiasmaticus, pars medialis
SO – optic striatum
SOC – supraoptic commissure
SOe – external supraoptic nucleus
SON – supraoptic nucleus
SONm – medial part of the supraoptic nucleus
SOv – ventral supraoptic nucleus
SSC – saline-sodium citrate buffer
SSO – subseptal organ
TAE –Tris-acetate EDTA
TEA/AA – triethanolamine/acetic anhydride
TH – tyrosine hydroxylase
TMA – tuberomammillary area
TnA – nucleus taeniae of the amygdala
TPH2 – tryptophan hydroxylase
tRNA – transfer ribonucleic acid
TSM – tractus septomesencephalicus
Tu – olfactory tubercule, tuberculum olfactorium
USV – ultrasonic vocalisations

UV – ultraviolet

V – volts

V1a – vasopressin receptor 1a

V1b – vasopressin receptor 1b

V2 – vasopressin receptor 2

V3 – third ventricle

VIP – vasoactive intestinal peptide

VLT – nucleus ventrolateralis thalami

VMH – ventromedial hypothalamus

VT1 – vasotocin receptor 1

VT2 – vasotocin receptor 2

VT3 – vasotocin receptor 3

VT4 – vasotocin receptor 4

ZI – zona incerta

Chapter 1 General Introduction

This chapter aims to provide a general overview of the background of this PhD thesis, as well as its overall aims.

1.1 Maternal behaviour

Reproduction is the ultimate goal of all life forms. Throughout evolution, a number of strategies have emerged to ensure the reproductive success of different species. Parental care is one of these strategies. Parental care is thought to have evolved on more than one occasion in many species when survival rates of offspring in the absence of care were low (Carlisle, 1982; Coleman et al., 1985; Pressley, 1980; Gross and Shine, 1981; for review, see Gross, 2005). The ways, in which animals care for their young, are very diverse. The level of parental care a species exhibits depends on factors including offspring maturity at birth and litter size. For humans and animals reliant on parental care in their infancy, the quality of parenting they receive can significantly influence both their immediate survival and their future success (Pittet et al., 2014b; Strathearn et al., 2001; Mills et al., 2011; Fox et al., 1988; Lovic et al., 2001; for review, see Strathearn, 2011). The level of involvement and parental investment between the two sexes differs across species, and in most species including most birds, the mother is the primary caregiver.

It is possible that it was the social aspect of maternal care that was the main driving force in its evolution. Early social interactions with the mother can have long-term effects on an individual's wellbeing and survival later in life. They are often crucial for the proper neurophysiological development of an animal. Various studies have shown that in many animals, limited contact

with the caregiver, as well as the lack of good quality parental care early in life, has a detrimental effect on social and cognitive skills, and even sexual behaviour (Cameron et al., 2008; Pittet et al., 2014b). Humans are no exception – failure to form a bond between mother and child can have long-term negative effects on the child's development and proper functioning in society (Strathearn et al., 2001; Mills et al., 2011; Fox et al., 1988). The damage that the absence of maternal care inflicts on the ability of an animal to mother their own offspring has been demonstrated in mammals such as rats (Lovic et al., 2001), as well as birds, e.g. quail (Pittet et al., 2014b). A significant behavioural change occurs when an animal 'becomes maternal'. Many females in which maternal behaviour has not been initiated either ignore the young of their species or respond negatively to them by avoiding or attacking them (Gibber et al., 1984; Richard-Yris et al., 1998) – a reaction likely related to territorial defence and possibly viewing the young as a threat. However, once maternal behaviour has been induced, individuals display care and protection for their young or for adoptees (Gandelman, 1973; Jakubowski and Terkel, 1980; Martín-Sánchez et al., 2015; Richard-Yris et al., 1983; Richard-Yris et al., 1987; Ruscio and Adkins-Regan, 2004; Scanlan et al., 2006; Swanson and Campbell, 1979). The onset and maintenance of maternal behaviour in both birds and mammals is often dependent on stimuli coming from the young and these stimuli can have strong physiological and behavioural effects (Edgar et al., 2011; Geissler et al., 2013; Mal'tsev, 1977; Orpen and Fleming, 1987; Pearson et al., 2011; Richard-Yris et al., 1998; Smotherman et al., 1976; Thayananuphat et al., 2011; Whitworth and Grosvenor, 1984).

In sheep, the bond between the mother and the lamb is facilitated by multisensory cues, primarily olfactory (Nowak et al., 2011). The neuroendocrine system in humans responds to a baby's cry (Swain et al., 2008). Vocalisations by young crocodiles directly before hatching act as a signal for the mother to open the nest (Vergne and Mathevon, 2008). Visual

and tactile stimulation from chicks induces rearing behaviour in incubating hens (Richard-Yris et al., 1998). Chicks also vocalise inside the egg prior to hatching, with the hen responding with vocalisations of her own (Tuculescu and Griswold, 1983). Some evidence in magpies (Soler et al., 2014) and zebra finches (Golüke et al., 2016) also suggests that some birds may use olfactory cues close to hatching to recognise their eggs, and fertilised quail eggs have been shown to emit different volatiles compared to non-fertilised (Webster et al., 2015). In fact, stimuli from young conspecifics can often be powerful enough to induce maternal behaviour in non-maternal and even virgin animals after sufficiently long contact with the young, and this has been demonstrated in both mammals (Gandelman, 1973; Jakubowski and Terkel, 1980; Martín-Sánchez et al., 2015; Swanson and Campbell, 1979) and birds (Richard-Yris et al., 1983; Richard-Yris et al., 1987; Ruscio and Adkins-Regan, 2004).

The changes in the brain associated with 'becoming maternal' are not only beneficial to the offspring but may provide direct benefits for the mother as well. There is evidence in rats that the maternal brain acquires enhanced learning skills (Kinsley et al., 1999) and protection against excitotoxicity (Vanoye-Carlo et al., 2008). In addition, it has been demonstrated that maternal behaviour acts on the reward system in the brain. Strikingly, given the choice, rat mothers choose pups over cocaine (Mattson et al., 2001; Pereira and Morrell, 2010). However, when the neuroendocrine system regulating maternal behaviour is disrupted and the offspring do not present a positive stimulus, this may lead to maternal neglect (Gammie et al., 2008; Bosch et al., 2007).

In the laboratory, maternal behaviour can be characterised by a number of indicators. In rats, these include building a nest, cleaning the young, moving them, assuming a specific crouching posture to feed them, as well as aggressive responses when the young are threatened. Maternal behaviour in chickens is characterized by the cessation of egg-laying, nest-building, sitting

on the eggs, displays of aggression, protection of the chicks once they hatch, crouching posture, gathering the chicks under the hen's wings and characteristic clucking. In precocial birds, unlike in mammals, it is possible to raise individuals in the complete absence of parental care. However, a study in quail showed that behavioural sex differences such as fearfulness and reaction to social isolation in the chicks may be shaped by maternal behaviour in this species. More specifically, chicks not brooded by a mother showed sex-related differences in tests for fearfulness while chicks brooded by a mother showed sex-related differences in their responses to social isolation tests. When developing in same-sex pairs, tonic immobility in non-brooded females lasted longer than in non-brooded males while there were no significant differences in mixed-sex pairs or in brooded chicks with either type of pair. Non-brooded chicks which developed in mixed-sex pairs emitted more distress calls than non-brooded chicks which developed in same-sex pairs while this pair effect was not evident in brooded chicks. By contrast there was no effect of sex, pair or interaction between the two in the social vocalisations emitted by non-brooded chicks in emergence and open-field tests while sex differences were present for brooded chicks with males emitting more distress calls than females in both tests. (Pittet et al., 2014a)

In addition, when left to rear their young, motherless quail mothers performed worse, than quail which were reared by their mother (Pittet et al., 2014b).

There is evidence that the brain regions and neuroendocrine mechanisms controlling social behaviour are conserved throughout evolution (Hara et al., 1990; Ivell and Richter, 1984; Sausville et al., 1985; for review, see Acher and Chauvet, 1995). This suggests that findings in the chicken and quail are likely to help us understand how maternal behaviour is regulated in other bird species as well as enabling comparisons across vertebrate taxa. Certain avian features are shared by other organisms, thus making birds a good model for comparative studies. Nesting and incubation behaviour are not unique to birds but exist in various species including crocodiles (the closest

living relatives of birds) (Ferguson and Joanen, 1982; Moore et al., 2010; Nelson et al., 2010), some snakes, including the python (Stahlschmidt and DeNardo, 2008), and in the platypus which is one of only two egg-laying mammals (Hughes and Hall, 1998). Similar to humans, many birds are socially monogamous, biparental and communicate through visual and auditory cues (for review, see Goodson et al., 2012). Indeed, our knowledge of modern birds has even been used to aid scientific predictions about their extinct ancient ancestors – the theropod dinosaurs (Grossi et al., 2014), although not yet in the context of maternal care.

Studies on 'broodiness' (a term often used to describe both incubation and rearing) and maternal behaviour are directly relevant to the farming industry. In third world countries where traditional poultry is still prevalent and an important part of the economy, farmers keep predominantly endogenous breeds of chickens (Farique et al., 2013; Adedeji et al., 2015; for review, see Chowdhury et al., 2014). These birds are often naturally more resistant to disease than commercial breeds and more capable of escaping predators, but they have a tendency to 'go broody' – cease egg-laying, incubate eggs and rear chicks – and while hens are used by farmers to hatch and rear chicks, unwanted broodiness still has a detrimental effect on egg production (Haunshi et al., 2011). Understanding the regulation of the behaviour may allow us in future to design ways to switch it on and off in indigenous breeds.

It has been shown in birds that the quality of parenting may increase with age while offspring abandonment decreases. This phenomenon is independent of maternal experience and is likely to be connected to hormone levels, which suggests an interesting hypothesis – that hormonal manipulation may enable birds to be better parents (Pittet et al., 2012). In conservation projects, abandoned young are sometimes successfully hand-reared (Waite et al., 2014) but because even in precocial bird species, maternal care is important for welfare and lifelong success (Pittet et al., 2012a; Pittet et al., 2014b),

birds reared by their mothers may be more viable and perform better later in life.

1.2 Brain areas of interest

The paraventricular nucleus of the hypothalamus (PVN), the medial preoptic area (MPOA, often designated as POM in birds), the supraoptic nucleus (SON), the medial bed nucleus of the stria terminalis (BnSTm) and the lateral septum (LS) are all brain areas which have been shown to be involved in maternal care across species (Broad et al., 1999; Da Costa et al., 1999; Domínguez et al., 2017; Katz et al., 1999; Kendrick et al., 1992; Meddle et al., 2007).

In sheep, parturition induced oxytocin release from the PVN (Da Costa, 1999; Broad et al., 1999), MPOA, BnST and LS (Broad et al., 1999), and in the PVN this release was increased even more by previous experience of birth (Broad et al., 1999). In the MPOA and BnST of sheep, oxytocin expression also increased during parturition and suckling (Kendrick, 1992), and oxytocin receptor mRNA was higher at parturition in the SON, MPOA and BnST of rat mothers compared to virgins and pregnant females (Meddle et al., 2007). On the other hand, bilateral lesions of the PVN disrupted nursing behaviour in the rabbit (Domínguez et al., 2017) and lesions of the medial PVN also impaired the initiation of maternal behaviour in the rat (Insel and Harbaugh, 1989). In mice, mothers had more neurones in the PVN expressing tyrosine hydroxylase (TH) - the rate-limiting enzyme for the production of dopamine - than virgins and males, and ablation of these neurones impaired, while optogenetic stimulation of TH expression facilitated maternal care (Scott et al., 2015). Further underlining the importance of the MPOA, in lactating

female rats, bilateral cuts in this brain area disrupted nest-building and pup-retrieval (Terkel et al., 1979), and in sheep, inactivation of the MPOA with the injection of lidocaine greatly impaired maternal behaviour during the first two hours postpartum (Perrin et al., 2007).

Evidence for the role of the SON in maternal care has been observed in the rabbit where the somal size of oxytocin-immunoreactive and vasopressin-immunoreactive neurones was larger on the first day postpartum compared to oestrus in both the PVN and SON (Caba et al., 1996). In addition, synapses in oxytocin neurones in the SON increased in the rat with gestation, parturition and suckling (Montagnese, 1987), and in virgin female rats behaving maternally, there was an increase in the number and size of dendrites in the SON, compared to non-maternal virgin females (Salm et al., 1988).

The LS is a major binding site for oxytocin although, surprisingly, oxytocin binding in the LS was found to be negatively correlated with juvenile alloparental behaviour - it was higher in species that do not display such behaviour (mice and meadow voles) than in species that do (prairie voles and rats) (Olazábal et al., 2006). However, vasotocin receptor 1a (V1a) density in the LS of mice was positively correlated with high incidence of postpartum licking and grooming of pups (Curley et al, 2012). Providing further evidence for the role of the LS in aspects of maternal behaviour, in the mouse LS, the tissue concentrations of the neurotransmitters glutamate and GABA were elevated in postpartum females relative to virgins (Zhao et al, 2014), and injections of a GABA receptor antagonist in this nucleus decreased maternal aggression in mice (Lee and Gammie, 2009).

These brain areas are similarly important in avian species. The POM, PVN and BnSTm were among the brain areas found to be activated in maternal turkey hens by the presence of chicks (Thayananuphat et al., 2011), and in the Thai hen, mesotocin-immunoreactive neurones which were likely to be

involved in rearing behaviour increased in number throughout the reproductive cycle in the PVN, POM and SON, with the highest numbers observed in birds exhibiting incubation and/or rearing behaviour (Chokchaloemwong et al. 2013).

Another forebrain area of interest is the nucleus of the commisurae pallii (nCPa), which is rich in gonadotropin-releasing hormone (GnRH) neurones and is involved mainly in sexual behaviours (Fraley and Kuenzel, 1993; Kang et al., 2006; Sartsoongnoen et al., 2012; Thayananuphat et al., 2007), but may have implications for the rearing of chicks (Chaiyachet et al., 2013a). In a study by Fraley and Kuenzel, sexual maturation in the male chick was characterised by a significant increase in GnRH-immunoreactive neurones in the nCPa (Fraley and Kuenzel, 1993), and in another study, c-fos immunoreactivity increased in the nCPa in male chickens after the display of appetitive sexual behaviours (Xie et al., 2010). GnRH mRNA was also found to be abundant in the nCPa of turkeys, and it was higher in layers than in non-laying or incubating hens (Kang et al., 2006). As demonstrated in the chicken, the number of GnRH-immunoreactive neurones in the nCPa was also highest in laying birds, compared to other reproductive stages (Sartsoongnoen et al., 2012), and there was a significant difference in GnRH immunoreactivity between non-rearing and rearing native Thai hens with non-rearing birds displaying more numerous cells positive for this peptide (Chaiyachet et al., 2013a). The nCPa has also been shown to be photosensitive, as GnRH mRNA expression in this nucleus was increased in the turkey by photostimulation with a 30-min or 90-min pulse of light at the start of the day (Kang et al, 2006). Photostimulation for 30 minutes 14 hours after first light also induced c-fos expression from nCPa GnRH neurones in this species (Thayananuphat et al., 2007). Interestingly, sporadic neurones positive for gonadotropin inhibitory hormone (GnIH), responsible for the inhibition of GnRH and reproduction, have also been found in the nCPa in the chicken (Zhang et al., 2017).

In addition to the forebrain areas mentioned above, the midbrain raphe nucleus which is a site of synthesis for monoamine neurotransmitters, including dopamine, noradrenaline, adrenaline and serotonin (5-HT), has also been shown to be important for maternal care (Barofsky et al., 1983a; Barofsky et al., 1983b; Harding and Lonstein, 2016; Holschbach and Lonstein, 2017; Pezzone et al., 1993; Yurino et al., 2001).

In lactating rat dams, neurotoxin lesions to the median raphe caused abnormal maternal behaviour including cannibalism and failure to retrieve pups, demonstrating that serotonergic cells in this nucleus may be necessary for maintaining maternal behaviour (Barofsky et al., 1983b). In addition, radiofrequency lesions or cuts in the median raphe in pospartum rat dams dramatically decreased retrieving and licking of pups, compared to controls (Yurino et al., 2001).

The dorsal raphe also appeared to be involved in maternal behaviour in a recent study in rats, as it exhibited significant neuroplasticity in response to motherhood - new cells born postpartum survived for a shorter period of time than those born during pregnancy and removal of pups caused cells to survive longer (Holschbach and Lonstein, 2017).

In mice, both fasting and injection of the orexigenic neuropeptide Y (NPY) to this nucleus lead to impaired maternal behaviour (Muroi et al., 2015). At the same time, neurotoxin lesions in the dorsal raphe serotonergic neurones which project to the hypothalamus led to a decrease in the suckling-induced prolactin secretion (Barofsky et al., 1983a), showing that the dorsal raphe may control this response. Juvenile female rats which had been alloparenting for 12 days had significantly quicker sensitisation to pups as adults, less anxiety and more tryptophan hydroxylase 2 (TPH2) - the rate-limiting enzyme in the synthesis of serotonin - expression in the dorsal raphe, compared to

females which had not been exposed to pups as juveniles (Harding and Lonstein, 2016).

The raphe nucleus has also been shown to be involved in stress in the rat. The dorsal raphe contains corticotropin releasing hormone (CRH) neurones that can be activated by nicotine, which is known to cause the release of stress hormones (Matta et al., 1997). In addition, the serotonin output determined by microdialysis in rats was increased in the median raphe by 5 minutes of handling stress, saline injection and forced swimming, and in the dorsal raphe by forced swimming (Adell et al., 1997). Electric shock induced c-fos from serotonergic neurones in the dorsal raphe, as well as release of serotonin from these neurones (Pezzone et al., 1993).

While many brain areas have been extensively studied in relation to maternal care, others have not received as much attention. In the course of this project, an attempt was made to establish the importance of some of these areas including the nCPa, the raphe nucleus and the BnSTl, in addition to expanding on existing knowledge of well-established neuronal networks in maternal behaviour in birds.

1.3 Hormones and neurotransmitters involved in maternal care

1.3.1 Nonapeptides

1.3.1.1 General overview and peripheral functions of nonapeptides

Nonapeptides are nine-amino-acid peptides with important roles in regulating egg-laying, water balance, learning and memory, and social behaviours

across taxa. Arginine vasotocin is the ancestral form from which other nonapeptides evolved through gene duplication (Heierhost et al., 1990; Nojiri et al., 1987). Vasotocin, which is found in non-mammalian species, and its mammalian orthologue vasopressin are involved in a range of behavioural responses in vertebrates including social recognition, aggression and stress (Bosch and Neumann, 2008, Bosch et al., 2010; Kuenzel et al., 2016). The other mammalian nonapeptide is oxytocin, and the oxytocin orthologue in birds, lungfish, reptiles, amphibians, and some marsupials is called mesotocin (Nojiri et al., 1987; Michel et al., 1993; Chauvet et al., 1981, Thayananuphat et al, 2011). The structure and functions of mammalian and avian nonapeptides are very similar (Tu et al., 1979).

In addition to being secreted by the pituitary gland, nonapeptides are also released from neurones directly into the brain where they act through their receptors to regulate a number of mammalian social behaviours, including maternal behaviour and pair-bonding (Bosch and Neumann, 2008; Bosch et al., 2010; Caughey et al., 2011; Walum et al., 2012; Insel and Hulihan, 1995; Insel et al., 1995; Klatt and Goodson, 2013a; Nephew and Bridges, 2008; Schneiderman et al., 2012).

Early studies found both vasopressin and oxytocin in hypothalamic extracts of a number of mammalian species including the human, the ox, the pig, the sheep, the rabbit and the rat (Lederis, 1961). Mesotocin and vasotocin in turn were shown to be present in the hypothalamus of a variety of birds including the common starling, the domestic pigeon, the collared turtle dove, the Japanese quail, the zebra finch, and the domestic mallard (Goossens et al., 1977). The localisation of neurones expressing these peptides is similar in different species. They are most abundant in areas of the social behaviour network in the brain, which suggests a conserved role in the control of social behaviours (Bons, 1980; Choy and Watkins, 1977; Goossens et al., 1977; Lederis, 1961; Ni et al., 2014). Although the distributions of oxytocin and vasopressin overlap, fractionation and subsequent assay for the two peptides

in beef pituitaries suggested that they may be secreted by separate cells (Labella et al, 1962). Immunohistochemistry showed the same separation of mesotocin and vasotocin in the common starling, domestic pigeon, collared turtle dove, Japanese quail, zebra finch and domestic mallard (Goossens et al., 1977). However, in the PVN of the chicken, double labelling for vasotocin immunoreactivity and mesotocin mRNA expression revealed that some neurones contained both vasotocin and mesotocin gene products (Barth et al., 1997).

The distribution of nonapeptide neurones throughout the brain shows some variation across species but they can be found in both mammals and birds in the PVN, SON, MPOA/POM and BnST (Bons, 1980; Choy and Watkins, 1977; Goossens et al., 1977; Lederis, 1961; Mikami et al., 1978; Ni et al., 2014). Figure 1.1 shows the neurosecretory vasotocin/mesotocin system in the brain of the duck (A) and quail (B) (Bons, 1980) and Figure 1.2 shows the mesotocin-immunoreactive neurones in the brain of the chicken (Kamrathok et al., 2017).

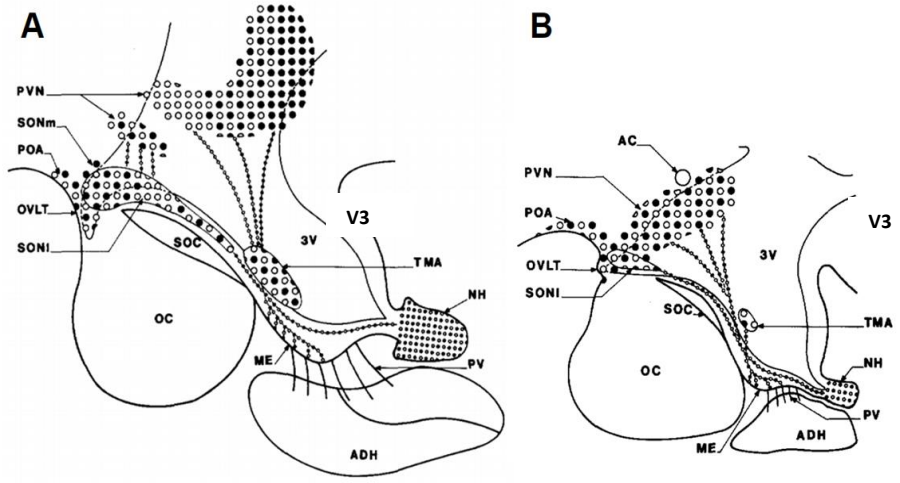

Figure 1.1 Schematic representation of the neurosecretory vasotocin and mesotocin systems in the hypothalamus of the duck (A) and quail (B).
Saggital view. Nuclear areas containing vasotocin (clear circles) and mesotocin (black circles). Connected circles represent vasotocin and mesotocin pathways. ADH (adenohypophysis); ME (median eminence); NH (neurohypophysis); OC (optic chiasma); OVLT (organum vasculosum of the lamina terminalis); PV (portal vessels); PVN (paraventricular nucleus); SOC (supraoptic commissure); SONl (lateral part of the supraoptic nucleus); SONm (medial part of the supraoptic nucleus); TMA (tuberomammillary area); V3 (third ventricle). Figure adapted from Bons, 1980.

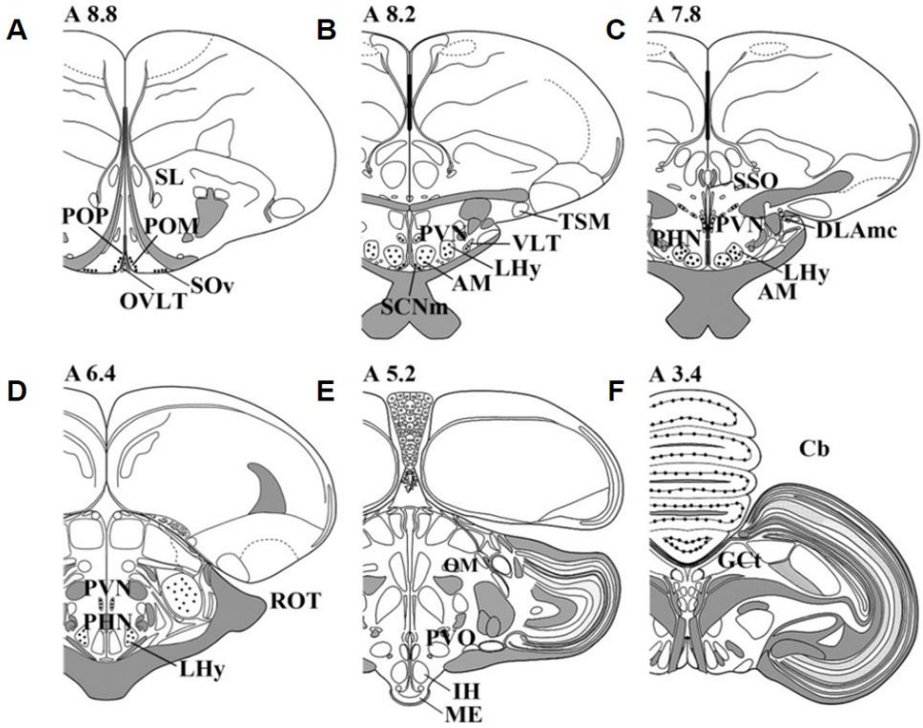

Figure 1.2 Schematic representation of the distributions of mesotocin-immunoreactive neurons (black dots) throughout the brain of the male native Thai chicken.
Coronal view. Illustrations were redrawn by Kamrathok *et al* (2017) from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988). AM (nucleus anterior medialis hypothalamic)*;* Cb (cerebellum); DLAmc (nucleus dorsolateralis anterior thalami, pars lateralis); GCt (substantia grisea centrlis); IH (nucleus inferioris hypothalamic); LHy (lateral hypothalamic area); ME (median eminence); OM (tractus occipitomesencephalicus); OVLT (organum vaculosum lamina teminalis); PHN (nucleus periventricularis hypothalamic); POM (nucleus preopticus medialis); POP (nucleus preopticus periventricularis); PVN (nucleus paraventricularis magnocellularis)*;* PVO (organum paraventriculare); ROT (nucleus rotundus)*;* SCNm (nucleus suprachiasmaticus*,* pars medialis); LS (lateral septum)*;* SOv (nucleus supraopticus, pars ventralis)*;* SSO (supseptal organ); TSM (tractus septomesencephalicus); VLT (nucleus ventrolateralis thalami)*.* Figure adapted from Kamrathok *et al*, (2017). The given coordinates (top of images) correlate to the Stereotaxic Atlas of the Brain of the Chick (Kuenzel and Masson, 1988), where ‘A’ followed by a number signifies a brain plate the given number of millimetres anterior to a zero reference plane.

The V1a, V1b and oxytocin receptors (OTRs) in the brain are responsible for mediating the central functions of mammalian nonapeptides (Thibonnier et al., 1996; Saito et al., 1995; , Ostrowski, 1998), while the avian nonapeptide receptors are VT1, VT2 (equivalent to the mammalian V1b), VT3 which is also known as oxytocin-like receptor (OTR-like) and is the main receptor for mesotocin, and VT4 (equivalent to the mammalian V1a) (Gubrij et al., 2005; Jurkevich et al., 2005; Leung et al., 2009; Tan et al., 2000). Oxytocin and vasopressin have been extensively studied. Vasopressin is known as the antidiuretic hormone for its involvement in osmoregulation. Both oxytocin and vasopressin can act in the periphery after secretion from the pituitary gland. Vasopressin controls water balance through interactions with its vasopressin receptor 2 (V2) in the kidney (Kaufmann et al., 2000) where it has been shown to facilitate water reabsorbtion (Dicker, 1953), in addition to increasing blood pressure (Petty et al., 1985). In a study in rats, water deprivation decreased the expression of the V2 receptor in the pituitary gland (Sharma and Chaturvedi, 2011). In birds, mesotocin and vasotocin are also involved in osmoregulation. One study in chickens demonstrated that during hemorrhage, mesotocin decreased while vasotocin increased (Bottje et al., 1989). In another study, water deprivation increased both vasotocin mRNA expression per neuron and the number of vasotocin mRNA-expressing neurones in the hypothalamus of the chicken, demonstrating that new vasotocinergic neurons were recruited, in addition to increasing expression from existing ones, to cope with osmotic stress (Chaturvedi et al., 1994). Osmotic stress has also been shown to affect the VT2 receptor in the pituitary gland of chickens. Water deprivation downregulated VT2 mRNA in this gland but VT2 immunoreactivity increased, suggesting that increased translation possibly shifted the balance towards more protein and less mRNA (Sharma et al., 2009).

In addition to also being involved in osmoregulation, oxytocin is known to cause uterine contractions in pregnant mammals, and estrogen has been shown to facilitate this effect in mice (Marrian and Newton, 1935; Parkes,

1930). Similarly, in birds, nonapeptides are involved in oviposition. Vasotocin content of the posterior hypophysis of hens was found to be significantly higher directly after oviposition, compared to any other time during the day (Tanaka and Nakajo, 1960). In addition, a study showed that the ovary in the chicken contained higher levels of mesotocin and vasotocin compared to plasma levels, and immunoreactive mesotocin in the ovary was highest at the time of oviposition, compared to five hours before or after, while vasotocin was highest five hours after oviposition, showing that both hormones varied with the ovulation cycle, albeit in different ways (Saito et al., 1990). Furthermore, mesotocin receptor binding in the oviduct in laying hens also changed before and after oviposition, with maximum binding capacity decreasing around the time of oviposition while binding remained unchanged within 24 hours in non-laying hens, providing further evidence for the role of mesotocin in oviposition (Takahashi and Kawashima, 2008). However, the role of mesotocin is mostly to support the actions of vasotocin. Mesotocin was found to increase the chicken oviduct's sensitivity to vasotocin, as an intravenous (IV) injection of mesotocin decreased the binding capacity (thus increasing binding affinity) of the vasotocin receptor in the uterus (Takahashi and Kawashima, 2008), and IV injection of vasotocin induced premature oviposition in hens, while injection of mesotocin had a smaller effect (Rzasa and Ewy, 1970).

1.3.1.2 Social functions of nonapeptides

1.3.1.2.1 Importance of the social environment

The importance of nonapeptides in social behaviour has been studied extensively in voles and in other mammalian species, including humans

(Kelly and Goodson, 2014; Insel and Shapiro, 1992; Rilling et al., 2017; Pedersen and Tomaszycki, 2012). Many of the effects of nonapeptides on behaviour can be influenced by social factors. A study in voles showed that oxytocin receptor distribution differed in different species of voles depending on their social organisation and levels of affiliative behaviours. In the highly parental prairie vole, there was a high density of receptors in the BnST - an area involved in maternal behaviour - while in the montane vole which generally does not exhibit affiliative behaviours, receptor density in areas connected to maternal behaviour was lower but increased before parturition (Insel and Shapiro, 1992). In pair-housed squirrel monkeys, while central vasopressin administration inhibited social behaviour in all treated subjects, central oxytocin administration increased aggression and sexual behaviour in the already dominant males but associative and marking behaviour in the subordinate males (Winslow and Insel, 1991). It was proposed that this may be related to different levels of testosterone in dominant and subordinate monkeys affecting oxytocin receptor densities in the brain (Winslow and Insel, 1991). Social organisation changed the effects of vasotocin on behaviour in birds as well, as central vasotocin infusions inhibited aggression in the territorial field sparrow but induced it in the colonial zebra finch, and in the violet-eared waxbill, which is more closely related to the zebra finch but is territorial, the effects on aggression were similar to those in the sparrow (Goodson et al., 1998).

1.3.1.2.2 Sex-specific actions of nonapeptides

Some of the actions of mesotocin and vasotocin are dependent on the sex of the individual according to studies in finches. Central administration of mesotocin increased, while mesotocin receptor antagonists reduced, time spent in large groups or with familiar partners in gregarious zebra finches.

This effect appeared to be female-specific and mediated by the lateral septum, as mesotocin administration in this area had significant effects, while treatment of a control area produced no effect. Mesotocin receptor distribution in different species of finches was correlated with their level of sociality in females. (Goodson et al., 2009)
Also in finches, central infusions of vasotocin enhanced aggression in both sexes but vasotocin antagonists were only effective in decreasing aggression in males, suggesting that natural levels of vasotocin may be more important for modulating aggressive behaviour in this sex (Goodson et al., 2015).
Knockdown through RNA interference of mesotocin and vasotocin in the PVN of zebra finches also produced diverse and sometimes sexually dimorphic behavioural effects – in the PVN, vasotocin knockdown significantly reduced gregariousness in both sexes and increased opposite-sex aggression in males but reduced it in females, while knockdown of mesotocin reduced gregariousness and pair-bonding in females only (Kelly and Goodson, 2014).
Oxytocin antagonists administered through intramuscular injection reduced pair-bonding in both sexes, but the effect was more pronounced in females, while in males treatment also reduced courtship song (Pedersen and Tomaszycki, 2012).

Sex-specific effects have also been observed in mammals. In the biparental marmosette monkeys, either oxytocin (a distinct variant of this hormone characteristic for the species) or vasopressin administered intranasally decreased latency to respond to infant stimuli after oral administration of their antagonists in a sex-specific manner, with oxytocin affecting males and vasopressin affecting females (Taylor and French, 2015).
These peptides do not readily cross the blood-brain barrier into the brain so the mechanism through which they achieve behavioural effects in some of the above examples is not clear, but such effects have also been observed in other studies cited later in this thesis. They may act on the brain through a ‘feed-forward’ loop – a mechanism also suggested by Modi *et al* in a study on

the effects of a peripherally administrated oxytocin receptor agonist on fear-induced freezing in male mice (Modi et al., 2016) or through some other mechanism involving binding to peripheral receptors and subsequent signalling to the brain.

1.3.1.2.3 Role of nonapeptides in social aggression

Nonapeptides have been implicated in the control of aggression but the mechanisms of their action are not entirely understood. Previous studies in rats suggest that vasopressin released within the central amygdala promotes maternal aggression, as release was higher in this region with the display of maternal aggression in a strain of rats showing high anxiety and high maternal aggression (Bosch and Neumann, 2010). Multiparous rats had enhanced aggression but lower oxytocin and OTR expression in the PVN on postpartum day 5 compared to primiparus dams, but this enhanced aggression and reduced oxytocin and OTR expression both disappeared by postpartum day 15, suggesting there may be a correlation between the two (Nephew et al., 2009). In avian species, previous studies suggest that mesotocin may be capable of both decreasing and facilitating aggression. In the violet-eared waxbill, peripheral injections of OTR antagonist significantly inhibited aggressive behaviours, but at the same time c-fos expression was lower in mesotocinergic neurones in the brains of these birds after displays of aggression (Goodson et al., 2015). Once again, the mechanism through which peripheral injections affected behaviour is yet unclear but in terms of the interpretation of these results, it was proposed that aggressive animals needed less HPA axis inhibition, even though mesotocin inhibition of the HPA axis may have been necessary for the display of aggression (Goodson et al., 2015). By contrast, individuals from a highly aggressive strain of Japanese quail were found to have significantly higher mesotocin gene expression and

number of large mesotocinergic neurones in the hypothalamus, compared to a less aggressive strain, suggesting that hypothalamic mesotocin may facilitate aggression in this strain (Maekawa et al., 2017).

1.3.1.2.4 Involvement of nonapetides in stress

The involvement of nonapeptides in the stress response is achieved through interactions with the hypothalamo-pituitary-adrenal (HPA) axis, and studies have shown that they play roles in both metabolic and social stress. In rats, both heat (Itoh, 1954) and noxious stimuli (Mirsky, 1955) caused the release of vasopressin from the pituitary gland, and immobilisation stress activated vasopressin neurones in the PVN in this species (Krisch, 1978). Peripheral administration of an estrogen receptor beta (ER-β) agonist in gonadectomised rats reduced anxiety (as measured by time spent in the open arms of an elevated plus maze) and corticosterone levels, but this effect was abolished when an oxytocin antagonist was administered centrally, suggesting that oxytocin was necessary for the downregulation of stress responses (Kudwa et al., 2014). In male rats subjected to chronic restraint stress, numbers of oxytocin-immunoreactive neurones also increased in the PVN (Li et al., 2016).

Like its mammamilan orthologue, vasotocin is also a major regulator of the stress response in birds, in both social and non-social context. Acute heat stress in the chicken increased plasma vasotocin but decreased mesotocin without affecting plasma osmolarity (Wang et al., 1989). In the same species, double-labelling for c-fos and vasotocin showed that parvocellular vasotocin neurones in the PVN became activated with both acute and chronic stress (Nagarajan et al., 2014). In the male song sparrow, vasotocin in the septum appeared to modulate the response to general stress stimuli, as central infusions of vasopressin to the lateral ventricle increased neuronal activation

in multiple brain areas when combined with a non-social stress factor and more limited areas when a social stressor was introduced (Goodson and Evans, 2004). There is evidence that some of vasotocin's effects on the stress response in birds are mediated by VT2 and VT4 receptors. VT4 in the pituitary of the chicken was found to be highly co-localised with corticotrophs, and VT4 mRNA was reduced significantly after acute restraint stress, while VT2 mRNA was increased (Selvam et al., 2013). By contrast, VT2 mRNA was significantly decreased in the pituitary gland of chronically stressed chickens and this corresponded to lower circulating levels of corticosterone in chronic stress, compared to acute stress (Kang and Kuenzel, 2014). In the same study, both chronic and acute stress caused a significant decrease in VT4 mRNA in the pituitary (Kang and Kuenzel, 2014). Finally, central administration of a VT4 receptor antagonist decreased the stress response after immobilisation stress in the domestic chicken (Kuenzel et al., 2016).

1.3.1.2.5 Involvement of nonapeptides in learning and memory

The role of nonapeptides in learning and memory appears to have been conserved throughout evolution as evidenced by the fact that an early nonapeptide found in nematodes (and accordingly named nematocin) has been implicated in associative learning in this species (Beets et al., 2012).

The effects of vasopressin and oxytocin on memory are sometimes opposite. Vasopressin has been shown to enhance and oxytocin to both enhance (in a social context only) and impair memory in mammals (Bohus et al., 1978; Kovács et al., 1979; Lande et al., 1972; Legros et al., 1978; Lukas et al., 2013; Weingartner et al., 1981; Wu and Yu, 2004). One proposed hypothesis for the biological reasons behind oxytocin's negative effects on memory is that it may be necessary for the "unlearning" of previous information in preparation for new learning (Freeman, 1997). In addition, as oxytocin has

been shown to impair the formation of long-term fear memories through interfering with memory consolidation (Hou et al., 2015), it may be necessary to prevent the long-term negative effects of fear.
One example of the memory-related effects of nonapeptides is a study in aging monkeys in which peripheral injections of vasopressin appeared to enhance while peripheral injections of oxytocin appeared to inhibit memory (Bartus et al., 1982). As mentioned previously, it is important to note that because of their chemical nature, nonapeptides do not readily cross the blood-brain barrier. Therefore, the mechanism through which their peripheral administration may influence memory processes is yet to be determined. It is possible that, in some cases, peripheral administration of nonapeptides triggers a feed-forward loop, in which binding to their peripheral receptors triggers nonapeptide release in the brain.

In rats, remembering a learned passive avoidance response was aided by central administration of vasopressin and impaired by central administration of oxytocin (Bohus et al., 1978). Also in rats, injected centrally with oxytocin, in the nucleus basalis of Meynert, spatial learning was impaired as determined by a water maze test, and this impairment was reversed by central administration of an oxytocin antagonist to the same nucleus (Wu and Yu, 2004). Interestingly, delivering an oxytocin receptor antagonist to the medial amygdala and LS of male rats and mice impaired their ability to retrieve a social memory but did not have an effect in non-social context, suggesting that oxytocin can also facilitate memory - but only in a social context - in this species (Lukas et al., 2013). Central microinjection of oxytocin in the hippocampus and dorsal raphe nucleus of rats impaired learned avoidance behaviour while injections of vasopressin facilitated it. However, injections of oxytocin in the dorsal septum also facilitated the behaviour (Kovács et al., 1979).

While oxytocin appears to both enhance and inhibit memory depending on context, the memory-enhancing capabilities of vasopressin have been well-

documented. Maze learning in mice was inhibited by central injection of puromycin (an antibiotic which inhibits protein synthesis) but this effect was rescued by subcutaneous injection of vasopressin (Lande, 1972). A vasopressin analog administered intranasally improved memory recall in both cognitively impaired and non-impaired humans (Weingartner, 1981). Vasopressin also aided various aspects of learning and memory, including attention, concentration, and recall in cognitively impaired human patients who received vasopressin, compared to controls (Legros, 1978).

Both V1a and oxytocin receptors have been implicated in mediating the memory effects of nonapeptides. Prenatal stress in rats caused sexually dimorphic deficits in the V1a receptor which led to changes in social memory, as prenatally stressed females had impaired social memory and lower V1a expression in the LS and BnST (Grundwald et al., 2016). Interestingly, acute stress facilitated social memory in prenatally stressed females and increased V1a expression in those two areas (Grundwald et al., 2016). However, in a study in adult Syrian hamsters, both oxytocin and vasopressin appeared to act on OTR rather than V1a to modulate social recognition, as central injections of oxytocin and vasopressin both prolonged the period of recognition of flank gland odours, oxytocin receptor agonists mimicked this, and oxytocin receptor antagonists blocked the effect, while V1a antagonists had no effect (Song et al., 2016).

Much like vasopressin, vasotocin has been shown to have memory-enhancing effects in birds. In day-old domestic chicks, peripheral administration of vasotocin prolonged short-term memory, thus facilitating the formation of intermediate memory (Gibbs and Ng, 1984), and central administration of vasotocin one minute after conditioning for a passive avoidance test also enhanced memory retention in young male chicks (Davis and Pico, 1984). Furthermore, both central and peripheral administration of vasotocin in domestic chicks 1 to 9 minutes after conditioning in an avoidance task enhanced memory retroactively (Davis et al., 1982).

1.3.2 Gonadal steroids

Parental and reproductive behaviours are strongly connected across taxa, as reproduction is normally inhibited in order for parental care to take place. This involves changes in gonadal sex steroids. Gonadal sex steroids, which are involved in the control of sexual behaviours and are secreted by the gonads, include testosterone, progesterone and, estrogen/estradiol (Andrew, 1963; Beach and Inman, 1965; Davidson, 1966; Hart, 1967; Hillsman et al., 2007; Moore and Zoeller, 1979; Panzica et al., 1996). While their main role is in reproduction, they have many functions, some of which are outlined below.

1.3.2.1 Effects on body weight and organ growth

Testosterone and estrogen can promote the growth of the sexual organs including the seminal vesicles, the prostate gland, the uterus and the vagina in rats (Dvoskin, 1947; Korenchevsky et al., 1936; Korenchevsky et al., 1937a; Korenchevsky et al., 1937b; Porter and Melampy, 1952). Treatment with testosterone has been shown to restore the normal weight of the liver, kidneys and heart which had decreased after castration (Korenchevsky et al., 1936; Korenchevsky et al., 1937a), and its effects on overall body weight could be both positive and negative, depending on the presence or absence of estradiol (Iwasa et al., 2017). In a previous study, chronic administration of testosterone caused a decrease in body weight in ovariectomized rats when no additional treatment was administered, but an increase when the ovariectomised rats were treated with estradiol (Iwasa et al., 2017). In the chicken, the growth of the comb was shown to be dependent on testosterone, progesterone, and estrogen with both estrogen and

progesterone reducing the increase in comb growth caused by testosterone, but the biggest combs being produced when a combination of the three hormones was delivered (Jaap and Robertson, 1953). These data indicate that the effects of gonadal steroids are not only focussed on the sexual organs but are widespread throughout the body and often depend on their interactions with each other.

1.3.2.2 Effects on sexual behaviour

Sex steroids play a crucial role in reproduction across taxa as has been extensively demonstrated in numerous studies (Beach and Inman, 1965; Davidson, 1966; Hart, 1967; Hillsman et al., 2007; Moore and Zoeller, 1979; Panzica et al., 1996 Dvoskin, 1947; Korenchevsky et al., 1936; Korenchevsky et al., 1937a; Korenchevsky et al., 1937b; Porter and Melampy, 1952).

In castrated male rough-skinned newts, testosterone replacement was necessary for the display of sexual behaviours facilitated by vasotocin (Moore and Zoeller, 1979). Testosterone, delivered through subcutaneous implants, stimulated both mounting behaviour and vasotocin expression in the brains of both sexual and unisexual whiptail lizards (Hillsman et al., 2007). Both of these examples not only provide evidence for the conserved functions of sex steroids but also highlight the link between them and nonapeptides. In rats, in castrated males with spinal transections, daily testosterone injections increased the number of reflex erections per minute while testosterone withdrawal reduced them (Hart, 1967), and the loss of sexual behaviour in castrated males of this species was completely recovered by testosterone implants in the MPOA (Davidson, 1966). Similarly, castration completely abolished while an intramuscular testosterone implant recovered sexual behaviours in male Japanese quail (Beach and Inman,

1965), and in senescent quail, administration of testosterone through subcutaneous implants recovered age-related loss of sexual behaviours completely, to a level similar to young, fully sexually active birds (Panzica et al., 1996). Testosterone delivered via intramuscular injection also facilitated copulation and aggression in the male domestic chick (Andrew, 1963).

Like testosterone, progesterone and estrogen are also important for sexual behaviour. Progesterone promoted lordosis in studies in rats and inhibited the negative effects that restraint stress had on lordosis (Frye et al., 2010; Hassell et al., 2011). Estrogen, acting through the ER-β receptor, was found to be essential for sexual behaviour in female mice (Antal et al., 2012), and estradiol promoted female sexual receptivity in the rat through interactions between the estrogen ER-α membrane receptor it activated and the metabotropic glutamate receptor 1a (Dewing et al., 2007). Interaction between ER-β and the glutamate 1a receptor has also been shown to promote sexual motivation in male quail (Seredynski et al., 2015).

1.3.2.3 Effects on other behaviour and functions

Interactions with other hormones allow sex steroids to be involved in a number of varied behaviours and functions in addition to their main role as regulators of the functions of the sexual organs. For example, testosterone appears to be involved in certain vasopressin-mediated memory functions, as castrated male rats have been shown to lose their sensitivity to the inhibiting effects of a vasopressin antagonist on social recognition (Bluthe et al., 1990). In mice, treatment with estrogen after the progesterone withdrawal observed at parturition had opposing effects on different aspects of maternal behaviour, with females showing shorter latencies to nursing their pups but also reduced nesting and pup retrieval (Murakami, 2016). In birds, along with prolactin, sex steroids are involved in the formation of the incubation patch

necessary for incubation. Either testosterone or estrogen synergised with prolactin, depending on whether the male or female of the species normally incubated the eggs, and progesterone appeared to play a secondary role in aiding the actions of estrogen (Johns and Pfeiffer, 1963; Jones, 1969). These examples once again demonstrate the importance of studying the interactions between sex steroids and other peptides in order to understand their role in the context of social interactions such as social recognition and parental behaviour, in addition to sexual behaviour.

1.3.2.4 Effects on the stress response

Apart from reproductive and social behaviours, sex steroids affect the stress response through interactions with the hypothalamus-pituitary-adrenal (HPA) axis, with testosterone most often acting to inhibit and estrogen to increase the stress response, although some exceptions for testosterone are also mentioned below. A number of studies have demonstrated that rats display sex differences in the secretion of corticosterone from the adrenal glands both at rest and in response to stress with females showing higher secretion levels than males (Critchlow et al., 1963; Handa et al., 2011). As with many instances of sexual dimorphism, these differences are established based on the sex-specific hormonal environment. Sex steroids have organisational effects on the HPA axis, as injections of testosterone in newborn female rat pups led to male-like stress responses in adulthood (Seale et al., 2005a) and the reverse was true when a female-like gonadal steroid environment was mimicked in male pups (Seale et al., 2005b). More specifically, the greater response to stress in female rats, coupled with the activation of neurones in the PVN, which is a known brain region involved in stress, has been shown to be facilitated by estradiol (Larkin et al., 2010), while testosterone was shown to downregulate the stress axis (Handa et al., 2011). In another

example of sex steroids and nonapetides working together, estradiol enhanced the anxiolytic effects of oxytocin in mice (McCarthy et al., 1996). In this study, central administration of oxytocin produced an anxiolytic effect by itself, but this effect was increased by estradiol pre-treatment. Pre-treatment with estradiol alone did not cause a significant anxiolytic effect as measured by the paradigm used (entries and time spent in the open arms of an elevated plus maze) but when this pre-treatment was combined with peripheral (intraperitoneal injection) administration of oxytocin, an anxiolytic effect was observed (McCarthy et al., 1996). The possible mechanisms through which a peripherally administered nonapeptide may act on behaviour without crossing the blood-brain barrier, such as a feed-forward loop, have already been mentioned in previous examples.

While the effects of sex steroids on the stress axis have been less studied in birds, some evidence exists for sex-specific differences in some elements of the HPA axis. Such differences are found in the mRNA expression of corticotropin releasing hormone (CRH) in the hypothalamus in starlings, with females showing higher expression levels than males when birds were kept outdoors (Dickens and Bentley, 2014). Interestingly, administration of testosterone caused greater activation of the HPA axis in the dark-eyed junco (Klukowski et al., 1997; Zysling et al., 2006) but testosterone and progesterone have both been shown to suppress plasma concentrations of corticosterone in cockerels (Nagra et al., 1965).

1.3.2.5 Effects on the oxytocin/vasopressin and mesotocin/vasotocin system

1.3.2.5.1 Effects on brain oxytocin/mesotocin

Oxytocin is affected by sex steroids in several brain areas as demonstrated by a number of studies. Estradiol treatment in the MPOA increased oxytocin mRNA expression in a study in female rats (Caldwell et al., 1989), while both estradiol and progesterone increased immunoreactive oxytocin levels in the same region, as detected by radioimmunoassay (RIA), but oxytocin perikarya decreased, suggesting that oxytocin synthesised in this area was quickly transported out of cells (Caldwell et al., 1988). Testosterone can also upregulate the oxytocin system, as in castrated male rats, both testosterone and estradiol increased oxytocin receptor binding and mRNA expression in the VMH (Bale and Dorsa, 1995). OTR binding in the VMH was also increased in ovariectomised females by treatments with estradiol (De Kloet et al., 1985; Johnson et al., 1989). As evidence for the importance of sex steroids in establishing the roles of behavioural peptides during development, a natural increase of oxytocin levels in the brains of rats during puberty, as determined by Northern blot, was counteracted by gonadectomy and restored by testosterone and estradiol treatment (Chibbar et al., 1990). Long-term treatment with estradiol also increased oxytocin levels in the PVN of mice (Patisaul et al., 2003), showing that oxytocin in this area was also susceptible to the influence of sex steroids. The expression of not only nonapeptides themselves but also their receptors can be affected by sex steroids. Treatment with estrogen after the progesterone withdrawal observed at parturition in mice increased the expression of oxytocin receptor in the medial amygdala (MeA) but reduced expression for both oxytocin and vasopressin receptors in the MPOA (Murakami, 2016). While the interactions

between sex steroids and mesotocin are largely unknown, in embryonic development in the chicken, the differentiation of both the vasotocin and the mesotocin system has been shown to be controlled by gonadal steroids (Robinzon et al., 1992).

1.3.2.5.2 Effects on brain vasopressin/vasotocin

The vasopressin/vasotocin system in the brain is another example of developing sexual dimorphism under the control of gonadal steroids. Vasopressin neurons in the LS are denser in male than in female rats and testosterone is necessary for establishing this difference on postnatal day 7 in normal development, in addition to being capable of causing male-like vasopressinergic innervation in females or neonatally castrated males as late as the third week of life (De Vries et al., 1983). There is evidence that similar processes occur in birds. As mentioned above, a study in embryonic chicks showed that the differentiation of the mesotocin and vasotocin system also appeared to be under the control of gonadal steroids in this species (Robinzon et al., 1992). The LS of birds, like that of rats, is sexually dimorphic with male birds displaying higher density of vasotocinergic neurones than females in this region and, as demonstrated in the zebra finch, the canary and the dark eyed junco, density decreased with castration and increased with testosterone treatment (Kimura et al., 1999; Voorhuis et al., 1988b). The same dimorphism has been demonstrated in the BnST in rats, zebra finches, canaries and juncos where castration abolished and treatment with testosterone restored vasopressinergic/vasotocinergic innervation (de Vries et al., 1984; Kimura et al., 1999; Plumari et al., 2004; van Leeuwen et al., 1985; Voorhuis et al., 1988b). Similar effects have also been observed in the POM in quail and dark-eyed junco (Plumari et al., 2004; Viglietti-Panzica et al., 1994).

As already mentioned above, sex steroids can also affect vasopressin receptors. In addition to estrogen reducing the expression of vasopressin receptors in the MPOA (Murakami, 2016), testosterone treatment increased the expression of VT4 - the avian equivalent of the V1a vasopressin receptor - mRNA in the POM, BnST and VMH of white-throated sparrows (Grozhik et al., 2014). While not sexually dimorphic, the vasotocin system in the PVN has also been shown to respond to sex steroid treatment. In female Japanese quail, both estradiol and testosterone administration increased vasotocin mRNA expression and vasotocin-immunoreactivity in the PVN by increasing the expression of existing neurons, although the total number of expressing neurons remained the same (Seth et al., 2004).

1.3.2.5.3 Effects on peripheral nonapeptide functions

Gonadal steroids can influence not only the central but also peripheral functions of nonapeptides. Along with acting directly on vasopressin expression, they can also act peripherally on vasopressin metabolism. A study in rats found that the plasma concentration and urinary excretion of vasopressin per 24 hours were higher in males than females, and in castrated males testosterone increased both plasma concentration and urinary excretion of vasopressin, while a combination of estradiol and progesterone decreased urinary excretion (Crofton et al., 1985). In the same study, vasopressin plasma concentration was decreased by progesterone only, while urinary excretion was decreased by both estradiol and progesterone in ovariectomised females (Crofton et al., 1985). In the brain of rats, hypovolemic shock activated oxytocin and vasopressin neurones in the PVN and the SON, and estradiol treatment was found to increase this activation (Mecawi et al., 2011), while a study in chickens demonstrated that testosterone treatment could increase the expression of the vasotocin

receptor VT2 in the chicken pituitary gland in the context of osmotic stress (Sharma and Chaturvedi, 2011). These examples suggest a role for testosterone and estradiol in the nonapeptide-mediated control of homeostasis. In addition, binding of oxytocin in the uterus, which is important for uterine contractions, is also induced by testosterone and estradiol in rats (Tribollet et al., 1990).

1.3.2.5.4 Involvement of estrogen receptors and aromatase in the interaction between gonadal steroids and nonapeptides

There is evidence in both mammals and birds that the effects of testosterone on vasopressin are sometimes achieved through testosterone's estrogenic metabolite estradiol following aromatisation in the brain, and not directly. In several studies, this mechanism appeared to be at work in the POM, BnST and LS of Japanese quail (Aste et al., 2013; Viglietti-Panzica et al., 2001) and the LS of rats (de Vries et al., 1986) where testosterone and estradiol seemed to have equivalent effects. In rats, both castration and inhibition of aromatase activity reduced oxytocin binding in the VMH and vasotocinergic innervation in the LS and BnST but not oxytocin innervation anywhere in the brain, while testosterone and estradiol increased oxytocin binding and vasotocin innervation but not vasotocin binding anywhere in the brain (Tribollet et al., 1990). Evidence from another study showed that the effects of testosterone on oxytocin receptor binding were also likely mediated by estradiol and dihydrotestosterone (DHT) in the rat VMH (Johnson et al., 1991).

Estradiol binds to estrogen receptors alpha (ER-α) and beta (ER-β) which have been found in areas of the behavioural network in the brain including the PVN, MPOA/POM, LS, BnST and SON, although their distribution varied between species (Axelsson et al., 2007; Kramer et al., 2005; Oyola et al.,

2017; Voigt et al., 2009). ER-β were co-localised with nonapeptide-expressing neurones in the PVN of mice (Patisaul et al., 2003). In Japanese quail, both ER-α and ER-β were found to be expressed in the LS, the dorsal thalamus, the nucleus taeniae of the amygdala, the nucleus intercollicularis, and in an area which extended from the POM to the caudal part of the tuberal hypothalamus, while ER-β only were expressed in the BnSTm (Voigt et al., 2009). In 9- and 12-day old quail embryos, the expression of ER-α was sexually dimorphic with females showing higher expression. However, the much more abundant expression of ER-β in sexually dimorphic brain regions including the POM and the BnSTm suggested that ER-β was the receptor involved in sexual differentiation in the brain in this species (Axelsson et al., 2007). Both of these receptors have been implicated in the regulation of nonapeptides by gonadal steroids. In the medial amygdala (MeA) of adult male mice, mRNA expression levels of OTR and V1a receptors were found to be highly correlated with ER-α mRNA expression, while oxytocin and vasopressin mRNA expression in the PVN was highly correlated with ER-β mRNA expression (Murakami et al., 2011). In support of the hypothesis that the effects of sex steroids are mediated by different receptors in different brain areas, another study in male mice found that the increase in oxytocin and decrease in vasopressin in the PVN (which, as was already established, contains ER-β) caused by implantation of a pellet containing estradiol, was abolished in ER-β knockout (KO) mice but in the same study estradiol significantly increased oxytocin in both wild type and KO mice in the MPOA (which is known to contain ER-α as well as ER-β) (Nomura et al., 2002). However, in both the SON and PVN of ovariectomised rats, ER-α-specific agonists increased vasopressin immunoreactivity while ER-β-selective agonists did not, suggesting that estradiol may act in these areas in the rat through afferent neurones containing ER-α receptors (Grassi et al., 2010). In humans, there was a strong decrease of ER-α and increase of ER-β immunoreactivity in vasopressin neurones in the dorsolateral SON of postmenopausal women, and these changes in both types of receptor were

suggested to be involved in the activation of vasopressin neurones in this brain area which occurs after the menopause (Ishunina et al., 2000).

1.3.3 Gonadotropin inhibitory hormone

The RFamide gonadotropin inhibitory hormone (GnIH) expressed in the PVN and the gonads of birds and mammals (Li et al., 2012; McGuire et al., 2011) is a known suppressor of reproduction (Bentley et al., 2006; Johnson et al., 2007) through its inhibitory actions on GnRH (Fraley et al., 2013; Osugi et al., 2004; Shimizu and Bédécarrats, 2010; Tobari et al., 2010; Tsutsui et al., 2000; Ubuka et al., 2008) and other reproductive hormones such as gonadotropin (Moussavi et al., 2013; Peng et al., 2016; Pineda et al., 2010; Qi et al., 2013; Ubuka et al., 2006), follicle stimulating hormone (FSH) and luteinising hormone (LH) (Fraley et al., 2013; Moussavi et al., 2012; Zhang et al., 2010). In addition, GnIH is affected by stress hormones and has been suggested to downregulate the activity of the hypothalamo-pituitary-gonadal (HPG) axis in many species (Calisi et al., 2008; Choi et al., 2017; Ernst et al., 2016; Kirby et al., 2009; Son et al., 2014; Ullah et al., 2017).

GnIH is also involved in the control of food intake as it has been shown to promote feeding in chickens (Tachibana et al., 2005), ducks (Fraley et al., 2013) and rats (Johnson et al., 2007). Evidence from sheep and rats (Clarke et al., 2012), as well as the Pekin duck (Fraley et al., 2013) also suggests that it may act as a molecular switch between reproduction and feeding.

In many species, GnIH shows seasonal variations and variations throughout the reproductive cycle. Most importantly, in the chicken, GnIH has been found to be higher in the brain during incubation and may be involved in the control of this behaviour (Ciccone et al., 2004a; Ciccone et al., 2004b), which

is of interest for this project and is discussed further in Chapter 5. The general fuctions of GnIH are outlined below.

1.3.3.1 Inhibition of reproductive hormones

As mentioned above, in addition to downregulating GnRH, in a number of species, GnIH downregulates gonadotropin (Moussavi et al., 2013, Qi et al., 2013; Ubuka et al., 2006; Pineda et al., 2010; Peng et al., 2016), follicle stimulating hormone (FSH), and luteinising hormone (LH) (Fraley et al., 2013; Zhang et al., 2010; Moussavi, 2012), all of which are involved in the control of reproduction. In mature male quail, two-week long continuous administration of GnIH through osmotic pumps implanted intraperitoneally decreased mRNA expression of gonadotropin common α subunit (a protein subunit shared by the heterodimer glycoprotein hormones including gonadotropin, LH, follicle-stimulating hormone and tyroid-stimulating hormone, each of which consist of two alpha and two beta chains) and LH β subunit (a protein subunit with a chain specific to LH and responsible for biological specificity), as well as plasma concentration of both LH and testosterone (Ubuka et al., 2006). This treatment also inhibited spermatogenesis and caused testicular apoptosis in adult males, while in juveniles it inhibited the normal development of the testes, and the rise in plasma testosterone and juvenile molting were also suppressed (Ubuka et al., 2006). Similarly, when cultures of diced cockerel pituitary glands were treated with GnIH, this treatment suppressed the release of LH and FSH, as well as mRNA levels of common α gonadotropin and FSH β subunit (Ciccone et al., 2004a). Central injection of GnIH also decreased plasma LH levels in the Pekin duck (Fraley et al., 2013) and a similar effect was achieved by intravenous injection of GnIH in free-living Gambel's white-crowned sparrows, where the inhibition of LH was shown to be very rapid - within 2 minutes (Osugi et al., 2004). The mammalian GnIH

orthologue (known as RFamide-related peptide, RFRP, with the most common form being RFRP-3) has been shown to have similar effects. Decreased circulating levels of LH and FSH in gonadectomised females, as well as both intact and gonadectomised males, after central injection of RFRP-3 were observed in rats (Pineda et al., 2010). Intravenous infusion of GnIH also blocked the increase of LH caused by administration of estrogen in ewes (Clarke et al., 2012).

The role of GnIH as an inhibitor of reproduction appears to be conserved throughout evolution, as actions similar to those in birds and mammals have been documented in other animals such as various species of fish (Choi et al., 2017; Moussavi et al., 2013; Peng et al., 2016; Qi et al., 2013; Spicer et al., 2017). A study on the patterns of expression of GnIH throughout the reproductive cycle in the common carp revealed that GnIH mRNA expression was decreased during reproduction and increased immediately after, while expression patterns of GnRH and LH were the opposite (Peng et al., 2016). Injection of synthetic GnIH inhibited GnRH (Qi et al., 2013) as well as the GnRH-induced increase in the LH β protein subunit and FSH β protein subunit in the goldfish (Moussavi et al., 2013) and the carp (Peng et al., 2016). Zebrafish GnIH orthologues have also been shown to reduce the expression of LH β subunit in pituitary explants and the variant GnRH3 in brain slices (Spicer et al., 2017). In the cinnamon clownfish increased GnIH mRNA levels after cortisol treatment corresponded to decreased GnRH mRNA levels as well as decreased circulating levels of LH and FSH (Choi et al., 2017).

1.3.3.2 Colocalisation and action on gonadotropin-releasing hormone neurones

GnIH neurones and fibres have been mapped in close proximity to GnRH neurones in a variety of species, supporting the hypothesis that GnIH inhibits GnRH neurones directly. In the frog, the distribution of GnIH neurones overlapped with that of GnRH neurones in the anterior preoptic area with some GnIH fibres in close proximity to GnRH cell bodies (Pinelli et al., 2015). GnIH and GnRH neurones and fibres were likewise co-localised in the hypothalamus of both house sparrows and song sparrows (Bentley et al., 2003) and RFRP-3 connections were also found close to GnRH neurones in bovines (Tanco et al., 2016). Even more convincingly, double labelling immunocytochemistry for GnIH and GnRH in the European starling not only showed that GnIH axons were in direct contact with GnRH cell bodies but, in addition, GnIH receptors were expressed in both types of GnRH cells (GnRH1 and GnRH2) in the brain in this species (Ubuka et al., 2008). In terms of its sites of action, unlike the mammalian RFRP-3, avian GnIH likely acts directly on the pituitary gland through its receptors as two GnIH receptors were localised to the pituitary in chickens (Ikemoto and Park, 2005).

1.3.3.3 Inhibition of reproductive behaviour

The ability of GnIH to inhibit sexual behaviours has been demonstrated in both mammals and birds (Bentley et al., 2006; Johnson et al., 2007). In female white-crowned sparrows, central infusions of GnIH rapidly inhibited copulation solicitation behaviour (Bentley et al., 2006). A similar effect on number of mounts and ejaculations was seen in male rats (Johnson et al.,

2007). In addition, RNA interference for the GnIH gene in white-crowned sparrows through the administration of small interfering RNAs in the third ventricle produced behaviours similar to territorial defence during breeding in males and stimulated agonistic vocalisations in both males and females, suggesting that interfering with the GnIH gene stimulated arousal in this species and that GnIH may be responsible for inhibiting sexual motivation (Ubuka et al., 2012). Effects of GnIH on sexual motivation have also been observed in mammals. In food-restricted female Syrian hamsters, an increase of the number of GnIH-immunoreactive neurones co-expressing c-fos was accompanied by a decrease in vaginal scent marking (a marker of sexual motivation) (Klingerman et al., 2011). A decrease of vaginal scent marking was also observed in females of this species when GnIH was administered centrally through chronic osmotic pumps (Piekarski et al., 2013).

1.3.3.4 Involvement of gonadotropin inhibitory hormone in stress

1.3.3.4.1 Effects of stress on gonadotropin inhibitory hormone expression

GnIH has been shown to be affected by corticosterone and has been implicated in downregulating the hypothalamo-pituitary-gonadal (HPG) axis in response to stress, although specific tissue responses and the direction of the response can vary across species (Calisi et al., 2008; Choi et al., 2017; Ernst et al., 2016; Son et al., 2014; Ullah et al., 2017; Kirby et al., 2009). Treatments with cortisol increased GnIH mRNA levels in cinnamon clownfish (Choi et al., 2017). Likewise, 24-hour treatment with corticosterone (CORT) increased GnIH expression in the hypothalamus of quail (Son et al., 2014),

and both chronic and acute immobilisation stress led to an increase in RFRP in the hypothalamus of adult male rats, and this was accompanied by a downregulation of the HPG axis (Kirby et al., 2009). By contrast, in the opportunistically breeding zebra finch, birds stressed by being restrained for 60 min after capture had significantly fewer GnIH-immunoreactive neurones than non-stressed birds (Ernst et al., 2016). However, stressed females had lower expression of the FSH β subunit in their pituitaries, suggesting that the lower number of GnIH-immunoreactive perykaria may have been due to rapid GnIH release in the female (Ernst et al., 2016). In the same study, GnIH expression increased in the testes of stressed males but not in the ovaries of stressed females, suggesting that there may be variations in how GnIH concentrations change in reaction to stress in different tissues (Ernst et al., 2016). It should be noted that the involvement of GnIH in the stress system is most likely context-dependent and tightly linked to the reproductive status of the animal. As mentioned above, GnIH-immunoreactive neurones increased in number in stressed house sparrows compared to controls, but this effect was only observed in spring (Calisi et al., 2008). Interestingly, some studies have suggested that GnIH itself can also activate the HPA axis. Mammalian forms of GnIH have been shown to increase plasma cortisol in rhesus monkeys (Ullah et al., 2017).

1.3.3.4.2 Mechanism for the actions of stress on gonadotropin inhibitory hormone

There is evidence that the effects of stress on GnIH levels may be mediated directly through glucocorticoid receptors. In adult male rats, 53% of RFRP-immunoreactive cells expressed glucocorticoid receptors and adrenalectomy prevented the stress-induced increase in RFRP expression, suggesting that adrenal glucocorticoids may downregulate the HPG axis during stress

through their effects on RFRP (Kirby et al., 2009). mRNA expression for glucocorticoid receptors (GRs) was also found in GnIH neurones in the hypothalamus of quail (Son et al., 2014). In the same study it was found that the GnIH precursor protein contains a region responsive to CORT which also included two glucocorticoid-responsive elements, the mutation of one of which abolished responsiveness to CORT (Son et al., 2014). CORT was found to promote the recruitment of GRs to the GnIH promoter containing this element (Son et al., 2014), providing further evidence in support of the hypothesis that GRs are responsible for the effects of stress on the expression of GnIH.

1.3.4 Prolactin

Prolactin is a hormone mainly produced by the anterior pituitary gland and its release can be stimulated by a number of hormones. In birds, specifically, vasoactive intestinal peptide (VIP), the primary function of which is as a vasodilator in the intestine, is a very powerful prolactin-releasing factor (Sharp et al., 1998; Dawson and Sharp, 1998; Chaiyachet et al., 2013b; March et al., 1994; Richard-Yris et al., 1998). Intravenous injections of VIP rapidly increased plasma prolactin in a number of bird species including domestic birds and wild species such as the white-crowned sparrow, dark-eyed junco, and Florida scrub-jay which were tested during the breeding season (Maney et al., 1999), as well as Mexican jays, Blue jays and zebra finches (Vleck and Patrick, 1999).

Prolactin receptors are widely distributed throughout the body and found in many different tissues such as the mammary gland, brain, liver, kidney, spleen, pancreas, heart, lung and uterus, as well as others (Roky et al., 1996; Simon-Holtorf et al., 2006; García-Caballero et al., 1996; Higashimoto et al., 2001; Nagano and Kelly, 1994; Ueda et al., 2011). Prolactin is most

known for its involvement in lactation in mammals (Bradley and Clarke, 1956; Kalyani et al., 2017) but it has many other functions in vertebrates and plays a role in stress, metabolism, and osmoregulation (Angelier et al., 2013; Jahn and Deis, 1986; Roberts, 1998).

Prolactin is known to be involved in the stress response although the direction in which plasma levels of this hormone change is context-dependent and possibly varies between species. Prolactin has been shown to downregulate the HPA axis in rats (Torner et al., 2001; Torner et al., 2002) and can be increased by acute stress in non-parental birds, including failed breeders (Angelier et al., 2013). However, acute and chronic stressors have also been shown to depress prolactin secretion in parental birds, suggesting that the prolactin stress response changes throughout the reproductive cycle and these variations may be involved in the decision between continuing the parental effort versus focusing the individual's efforts on its own survival (Angelier et al., 2007; Angelier et al., 2013; Chastel et al., 2005; Gustafson et al., 2017; Kalyani et al., 2017; Schmid et al., 2011). In one specific example in a sea bird - the Manx shearwater - prolactin levels decreased in response to acute stress during incubation and mid chick-rearing but increased with stress during late chick-rearing and in non-parental birds (Riou et al., 2010). Furthermore, acute restraint stress elevated plasma prolactin in the turkey (el Halawani et al., 1985) but it decreased it in the rock pigeon (Angelier et al., 2016). Krause *et al* (2015) observed in white-crowned sparrows that prolactin levels did not change as a result of acute restraint stress at any point in the reproductive cycle. Further examples in mammals demonstrate that the relationship between prolactin and stress can be complex. Handling stress, anaesthesia and blood sampling increased prolactin in male rats (Euker et al., 1975), and in humans, prolactin was elevated by various stress conditions including surgery and exercise (Noel et al., 1972). However, in ovariectomised oestrogen-treated female rats, while prolactin increased consistently in response to restraint stress in the morning, in the afternoon the response depended on the already present prolactin level - if it was low,

response was as in the morning but if it was high the response was reversed and prolactin levels decreased (Smith et al., 1977). In the same study there was no response in ovariectomised rats not treated with oestrogen which suggests that oestrogen is necessary to mediate this response (Smith et al., 1977).

Prolactin is strongly implicated in incubation. The significance of prolactin during the period of incubation has been demonstrated in many studies (Cherms et al., 1962; Criscuolo et al., 2006; Hall, 1991), although the mechanism of its action is still not entirely clear. It has been suggested that, rather than directly inducing incubation behaviour, prolactin acts by lowering the threshold for environmental stimuli, like signals from the nest and eggs – in other words, the presence of high levels of prolactin may make an animal more responsive to the presence of a nest or a clutch of eggs which would have otherwise not provoked a change in behaviour by themselves (Chaiyachet et al., 2013b; March et al., 1994; Richard-Yris et al., 1998). Prolactin is one of the hormones responsible for the formation of the incubation patch in avian species, as well as nest-protecting behaviour (Hutchison et al., 1967; Höhn and Cheng, 1965; Jones, 1969; Book et al., 1991; Massaro et al., 2007; Ohkubo et al., 1998). One study has suggested that lack of incubation behaviour in White Leghorn chickens may be due to incorrect processing of prolactin receptor in the hypothalamus (Ohkubo et al., 1998).

1.3.5 Monoamine neurotransmitters

The monoamine neurotransmitters dopamine, serotonin (5-HT), adrenaline and noradrenaline are synthesised in the brain and are involved in a number of functions and processes across species including cognition, behaviour, learning and stress (Bäckström et al., 2018; Díaz-Mataix et al., 2017; Hartline

et al., 2017; Joling et al., 2018; Kubikova et al., 2010; Steele et al., 1979; Tokarev et al., 2017). The raphe nucleus is one of the important brain areas where these compounds and their precursors are synthesised (Aghajanian et al., 1967; Challet et al., 1996; Kostowski et al., 1968; Okado et al., 1992; Reinoso-Suarez and Morgane, 1976; Saavedra et al., 1976; Versteeg et al., 1976).

1.3.5.1 General overview and peripheral functions

The neurotransmitter dopamine can be converted to noradrenaline and then to adrenaline, as demonstrated in some early studies. Infusions of dopamine in various tissues in the cat caused an increase in noradrenaline (Pennefather and Rand, 1960). Dopamine was found in the brain of the rabbit at concentrations similar to those of noradrenaline, and dopamine concentrations were increased by the injection of its precursor 3,4-dihidroxyphenilalanine (DOPA) (Carlsson et al., 1958). Dopamine, adrenaline, and noradrenaline have been documented in the brains of numerous species of mammals and birds including the cow, sheep, pig, dog, cat, rat, rabbit, guinea pig (Bertler and Rosengren, 1959), duck, chicken, pigeon and finch (Juorio and Vogt, 1967). Dopamine concentrations in the brain of the above-mentioned mammalian species were found to be of the same order of magnitude as those of noradrenaline, and dopamine was mostly found in brain areas such as the caudate nucleus where there was little noradrenaline, while in places where dopamine was low, noradrenaline concentration was high. This emphasises the fact that dopamine has its own function in addition to its role as a precursor. (Bertler and Rosengren, 1959) In the chick, the highest amount of dopamine was found in the cerebral hemispheres, while noradrenaline was ubiquitously expressed with relatively high concentrations in the cerebellum (Kobayashi and Eiduson, 1970), and it

was the major catecholamine (a group of dihydroxybenzene monoamines which includes, dopamine, adrenaline and noradrenaline) in the chick neurohypophysis (Enemar and Ljunggren, 1968).

Another important monoamine neurotransmitter is serotonin (5-HT) which is crucial for normal brain function and its release from brain cells can be triggered by the adrenergic blocking agent reserpine, as was demonstrated in male rabbits (Pletscher et al., 1956). 5-HT is mostly synthesised in the raphe nucleus as shown in a number of studies. Lesions in the raphe nucleus in the rat (Kostowski et al., 1968) and the cat (Reinoso-Suarez and Morgane, 1976) caused a depletion of 5-HT in the forebrain, while electrical stimulation to this area in the rat caused 5-HT release in the forebrain (Aghajanian et al., 1967). 5-HT neurones were also observed in the chick raphe nucleus as early as embryonic day 4 (Okado et al., 1992), and they were mostly found in the raphe nucleus in the pigeon, although they were also present in the hypothalamus (Challet et al., 1996).
Not only 5-HT but noradrenaline and dopamine were also present in the raphe nucleus in the rat, although while noradrenaline concentration was high, that of dopamine was fairly low (Saavedra et al., 1976; Versteeg et al., 1976).

Monoamines have various physiological effects on body temperature (Marley and Stephenson, 1970; Scott and van Tienhoven, 1974), feeding (Mahzouni et al., 2016; Zhang et al., 2017) and osmoregulation (Bolton and Bowman, 1969; Burn and Rand, 1958; Ginzel and Kottegoda, 1953; Hornykiewicz, 1958; Swan, 1949), and can also be involved in pregnancy and birth in mammals (Abrahams and Pickford, 1956; Schriefer et al., 1980). For example, infusions into the brain of noradrenaline lowered the body temperature of chickens (Marley and Stephenson, 1970). Serotonin had an anorexigenic effect in chicks (Zhang et al., 2017). 5-HT, adrenaline and noradrenaline have been shown to have a vasoconstrictor action in species such as dog and cat but adrenaline, along with dopamine has been

demonstrated to have both depressor and pressor action on the cardiovascular system of the rabbit and guinea pig, and the change appeared to be triggered by the presence of noradrenaline (Burn and Rand, 1958). Administration of dopamine to pregnant rats prolonged gestation (Schriefer et al., 1980). Disruption of the monoaminergic systems in the brain, specifically the dopamine and 5-HT systems, has been found to contribute to neurodegenerative disorders such as Parkinson's disease (Buddhala et al., 2015; Bäckström et al., 2018; Joling et al., 2018).

1.3.5.2 Roles of monoamines in reward

As mentioned previously, maternal behaviour is rewarding to the mother, which has been illustrated by the finding that rat mothers showed a preference for pups over cocaine (Mattson et al., 2001; Pereira and Morrell, 2010). Therefore, the brain neurotransmitters involved in the control of reward may be of significant importance for maternal behaviour, as in the absence of this rewarding aspect, there may not be sufficient motivation for the behaviour, which may lead to its disruption.

The importance of dopamine as part of the reward system has been well-documented. Lesions to the dopaminergic pathway in the nucleus accumbens in the rat's brain decreased preference for locations where a heroin reward was received, demonstrating that the mesolimbic dopaminergic system is involved in heroin reward (Spyraki et al., 1983). Furthermore, intraventricular injection of dopamine receptor antagonists significantly reduced self-stimulation (electric stimulation delivered directly to the brain by pressing a lever) in rats (Lippa et al., 1973), and a further study revealed that dopamine receptor antagonists were capable of producing this effect without causing sedation, unlike adrenaline receptor antagonists which also decreased self-stimulation but this was likely due to their sedative effect,

evidenced by decreased locomotor activity and decreased rearing behaviour (Rolls et al., 1974). D2 receptors specifically are known to have inhibitory effects and, as would be expected, D2 antagonists induced while agonists reduced lever pressing for intracranial reward in rats (Katz, 1979). Dopamine receptors directly, rather than levels of dopamine or noradrenaline, appear to be responsible for reward, as it has been shown in rats that the dopamine receptor stimulant apomorphine induced rats to press a lever to receive the reward even when dopamine and noradrenaline levels were depleted (Baxter et al., 1976). In humans, dopamine transporter availability, assessed by positron emission tomography (PET), and reward-related neural response, assessed by functional magnetic resonance imaging (fMRI), were recorded in healthy individuals and patients with schizophrenia, depression and cocaine addiction, and in all participants, healthy and otherwise, dopamine transporter correlated positively with the anticipation of reward in the nucleus accumbens (Dubol et al., 2017).

The involvement of the dopaminergic system in reward signalling has also been demonstrated in birds. In laying hens, the D2 antagonist haloperidol injected 30 min before a conditioned cue signalling a reward, diminished anticipatory responses compared to saline control, suggesting that dopamine signalling may be involved in reward processes in this species (Moe et al., 2011). This was supported by another study which showed that in both Red Jungle Fowl and domesticated white layer cross chickens, conditioned to expect mealworms 25 seconds after a red light stimulus, both anticipatory behaviours after the stimulus and foraging behaviour after the reward were reduced by haloperidol (Moe et al., 2014).

In the context of rewarding and aversive stimuli, monoamines also play a role in learning. A study in mice demonstrated that 5-HT neurones in the dorsal raphe nucleus may be involved in cognitive flexibility as they became activated during learning by both positive and negative stimuli (Matias et al., 2017). Noradrenaline was found to be important for the long-term retention of

a learned behaviour in rats (Atucha et al., 2017). In birds, monoamines may be important for song learning, as suggested by studies in species such as the zebra finch (Gadagkar et al., 2016; Kubikova et al., 2010), the Bengalese finch (Hoffmann et al., 2016), and the European starling (DeVries et al., 2015; Riters et al., 2014).

1.3.5.3 Roles of monoamines in other behaviour

Sexual behaviours, aggression, anxiety and stress can also be controlled by monoamines, in particular dopamine and 5-HT. For example, sexual behaviour in the male Japanese quail was promoted by D1 agonists and D2 antagonists and inhibited by D1 antagonists and D2 agonists (Balthazart et al., 1997b; Kleitz-Nelson et al., 2010). 5-HT has been implicated in aggression in varying ways depending on the species and context. Its depletion in the forebrain of rats as a result of lesions in the raphe nucleus led to higher incidence of mouse killing (Grant et al., 1973) but in mice, lesions in the raphe nucleus which caused decreased forebrain 5-HT, decreased aggression provoked by prolonged isolation (Kostowski and Valzelli, 1974). Intracerebroventicular administration of 5-HT receptor agonists also reduced maternal aggression in rats (De Almeida and Lucion, 1994). Similarly varied effects were found in birds. High aggression was correlated with reduced 5-HT activity in the brain of the brown anole (Hartline et al., 2017) but aggressive White Leghorn chickens became more aggressive after injection of a 5-HT agonist (Dennis et al., 2013). Studies in mice and rats have also demonstrated that 5-HT and noradrenaline were both involved in the control of anxiety in rats and mice and had an anxiolytic effect (García-García et al., 2017; Pereira et al., 2017).

Of particular interest for this project is the involvement of monoamines in maternal behaviour. Studies in rats and mice have shown that dopamine, 5-HT and noradrenaline may be necessary for maternal care in mammals (Rosenberg et al., 1977; Steele et al., 1979; Calamandrei et al., 1992; Rothlin et al., 1922; Cox et al., 2011; Byrnes et al., 2002; Tanaeva et al., 2012; Curry et al., 2013; Wilkins et al., 1997). Their role in birds, however, has not been established.

1.4 Aims of thesis

The overarching aim of this PhD project was to investigate the neuropeptides and neurotransmitters involved in the control of maternal behaviour, including incubation and chick-rearing in birds, using the domestic chicken and the quail as model organisms. In addition, another aim was to examine the possible involvement of brain areas such as the BnSTl, nCPa and the raphe nucleus, which have received little attention from previous studies on maternal care. Specific objectives included:

1. Examining the effects of sex steroids on mesotocin in the PVN and BnSTl of birds, which effects, to the author's knowledge, had not been established prior to this project. Sex steroids promote sexual behaviours and their plasma concentrations are usually high during the period of reproduction and decrease during the parental period. However, studies in mammals have shown that they can elevate oxytocin in the brain. This is interesting because high levels of oxytocin promote the display of maternal behaviour. This study is a step towards better understanding how the balance between the two opposite behaviours - sexual and maternal behaviour - is achieved, as it provides information on how the key hormones involved in both behaviours interact. Given how closely-related oxytocin and mesotocin

are, it was considered reasonable to expect similar effects of sex steroids on mesotocin in birds as have been found for oxytocin in mammals. Therefore, the hypothesis that acute treatment with sex steroids would increase mesotocin mRNA expression and immunoreactivity in the brain behaviour network of the chicken was tested (Chapter 3). Juvenile chickens were used for this study as they do not yet produce their own sex steroids which may have interfered with the study, and they were a cheaper alternative to using gonadectomised adults.

2. Investigating the acute effects of sex steroids on the vasotocin system in the PVN of thefemale chicken, which are not known in this species, and in addition, establishing the sex steroid effects on the BnSTl, previously unexamined in this context in any avian species (Chapter 3). Sex steroids have been shown to stimulate the vasotocin system in the PVN and BnSTm of quail so similar effects in the chicken were expected. Therefore, the hypothesis that acute treatment with sex steroids would increase vasotocin mRNA expression in the PVN and BnSTl of female chickens was tested. The BnSTl, like the BnSTm, is part of the BnST but it is not very often studied by itself so findings about it will complement what is already known from studies on the BnSTm. As stated above, using juvenile birds which had not started producing their own sex steroids was a cheaper alternative to using gonadectomised adult birds.

For this and the above aims, while the age of the birds should be taken into consideration when interpreting results, this is the first study of its kind in the chicken and results from it can serve as basis for future work, especially since they corresponded well to previous data from adult gonadectomised quail.

3. Characterising the changes in the mesotocinergic and vasotocinergic systems in the brain of the domestic hen throughout the reproductive cycle in areas of the brain behaviour network

previously implicated in maternal care such as the PVN, POM, SON, and BnSTm, as well as the BnSTl, the role of which was not known (Chapter 4). As oxytocin and vasopressin, as well as mesotocin, have been demonstrated to be upregulated in the brains of mammals and birds respectively during the maternal period, it was hypothesised that both mesotocin and vasotocin mRNA expression, immunoreactivy and receptor binding would be higher in areas of the brain behaviour network in incubating and rearing hens, compared to laying hens. Unfortunately, vasotocin immunoreactivity and receptor binding could not be tested due to the lack of suitable antibody/ligand.

4. Characterising the changes in the expression of the RFamide GnIH for which only limited studies exist on its involvement in the control of incubation in birds and maternal care in birds and mammals (Chapter 5). GnIH has been shown to downregulate sexual behaviours and to be upregulated during incubation in birds. Therefore, it was hypothesised that GnIH mRNA and immunoreactivity would be higher in the brains of incubating and rearing hens compared to laying hens.

5. Characterising the changes in mRNA expression of prolactin and the D2 dopamine receptor in the pituitary gland of the domestic hen throughout the reproductive cycle and investigating a possible relationship between the two. The D2 receptor has been implicated in downregulating prolactin and prolactin has been demonstrated to increase in plasma during incubation in birds. The expression of these two genes has been found to be inversely correlated in the turkey. Therefore, it was hypothesised that a similar relationship would be present in the chicken with prolactin mRNA expression increasing with incubation in the pituitary gland while D2 expression decreased (Chapter 6).

6. Characterising the changes in expression of monoamine neurotransmitters in the raphe nucleus of the domestic hen throughout the reproductive cycle. Monoamine neurotransmitters including

dopamine, serotonin and noradrenaline have been shown to promote maternal behaviours in mammals and the raphe nucleus appears to be involved in this control. Therefore, the hypothesis that monoamines would increase in concentration in this brain area during incubation and rearing in the chicken was tested (Chapter 6).

7. Investigating the brain areas which become activated in the brain of precocial birds during interactions with chicks using the quail as a model organism. A number of brain areas including the PVN, MPOA/POM, SON, LS and BnST have been demonstrated to be involved in and become activated during maternal behaviour in both mammals and birds, and introduction to young conspecifics has been shown to induce maternal behaviour in a number of animals after a period of cohabitation. Therefore, using the quail as a model (since, as previously explained, there was no access to chickens for this experiment), the hypothesis that areas of the brain behaviour network which are known to be important for maternal care would become activated in birds in the presense of young vs adult conspecifics after habituation to young was tested (Chapter 7).

Chapter 2 General Materials and Methods

2.1 Materials

2.1.1 General chemicals

Item	Company	CAS or catalogue number
Acetic anhydride	Sigma-Aldrich Company Ltd., UK	A6404
Acetone	Sigma-Aldrich Company Ltd., UK	439126
Acetonitrile	Fisher Scientific, UK	83640.320
Activated charcoal	Sigma-Aldrich Company Ltd., UK	5105
Agarose	Pomega, Inc., UK	V3121

Albumin, bovine	Sigma-Aldrich Company Ltd.,	A-7906
Ampicillin powder	Sigma-Aldrich Company Ltd., UK	A2804
Ammonium acetate	Sigma-Aldrich Company Ltd., UK	73594
Ammonium Chloride	Scientific Laboratory Supplies	CHE1150
Benzoyl Chloride	Sigma-Aldrich Company Ltd., UK	B12695
Bromophenol Blue	Sigma-Aldrich Company Ltd., UK	318744
Caffeic acid	Sigma-Aldrich Company Ltd., UK	C0625

CC Mount	Sigma-Aldrich Company Ltd., UK	C9368
Charged membrane	Roche Diagnostics Ltd., UK	11209272001 (foreign trade community code)
Chicken serum	Sigma-Aldrich Company Ltd., UK	C5405
Chloroform	Scientific laboratory supplies	CHE1570
Chloroform: isoamyl alcohol	Sigma-Aldrich Company Ltd., UK	C-0549
Corn oil	Sigma-Aldrich Company Ltd., UK	C8267
Deionised formamide	Pomega, Inc., UK	H5052

Denhardt's solution	Sigma-Aldrich Company Ltd., UK	D2532
Desoxyribonucleic acid (DNA), salmon testes	Sigma-Aldrich Company Ltd., UK	D-1626
DNAzol	Fisher Scientific, UK	10503027
Developer (Phenysol)	Ilford, UK	1757635
Dextran sulphate	Sigma-Aldrich Company Ltd., UK	D8906
3,3'-diaminobenzidine (DAB)	Sigma-Aldrich Company Ltd., UK	D7304
DH5-alpha competent cells	Thermo Fisher Scientific, UK	18265017

Dithiothreitol	Sigma-Aldrich Company Ltd., UK	D9779
Di-sodium hydrogen orthophosphate	Fisher Scientific, UK	S/4520/63
Diethylpyrocarbonate (DEPC)	Sigma-Aldrich Company Ltd., UK	D5758
DPX mounting medium	Sigma-Aldrich Company Ltd., UK	6522
Ethanol	Fisher Scientific, UK	E/0650DF/17
Ethylenediaminetet-raacetic acid (EDTA)	Sigma-Aldrich Company Ltd., UK	60-00-4
Ethylene glycol	Sigma-Aldrich Company Ltd., UKUK	324558
Fixative	Ilford, UK	1984262

Gelatine	BDH (Merck Ltd.), UK	1040700500
Gel emulsion (K5)	Ilford, UK	1355127
Glycerol	Sigma-Aldrich Company Ltd., UK	G5516
Hematoxylin “Z”	Cell Path Ltd.	RBA-4201-00A
Heparin sodium	Leo Laboratories Ltd.	PL00431149
HCl (hydrochloric acid) solution	Fluka Analytical, UK	35335
Hydrogen peroxide solution	Sigma-Aldrich Company Ltd., UK	H1009
L-Ascorbic Acid	Sigma-Aldrich Company Ltd., UK	A-7631

LB Agar	Sigma-Aldrich Company Ltd., UK	A5306
LB Broth	Sigma-Aldrich Company Ltd., UK	L7025
Magnesium chloride	Sigma-Aldrich Company Ltd., UK	M8787
Methanol	Fisher Scientific, UK	67-56-1
Mounting medium	VWR International, LLC	361603E
Nescofilm (Parafilm)	Bemis NA, Neenah, WI	12378039
Nickel Ammonium Sulphate	Fisher Scientific, UK	A/5800/53
Normal Goat Serum	Sigma-Aldrich Company Ltd., UK	G6767

Nuclear Fast Red	Sigma-Aldrich Company Ltd., UK	N3020
Paraformaldehyde	Sigma-Aldrich Company Ltd., UK	158127
Phenol: chloroform isoamyl alcohol	Sigma-Aldrich Company Ltd., UK	P3803
Potassium chloride	Fisher Scientific, UK	P/4240/60
Potassium dihydrogen orthophosphate	Fisons Scientific Equipment, Ltd, UK	P/4800/53
Propan-2-ol (Isopropanol)	Fisher Scientific, UK	P/7500/17
Scinti Safe 3 Scintillation fluid	Fisher Scientific, UK	sc/9205/21

Sodium acetate	Sigma-Aldrich Company Ltd., UK	S2889
Sodium chloride	Fisher Scientific, UK	s/3160/60
Sodium dihydrogen orthophosphate dehydrate	Fisher Scientific, UK	s/3720/53
Sodium hydroxide	Fisher Scientific, UK	S/4920/53
Sodium tetraborate	Sigma-Aldrich Company Ltd., UK	221732
Sucrose	Fisher Scientific, UK	s/8600/60
SYBRsafe	Thermo Fisher Scientific, UK	S33102

Toluidine blue	Sigma-Aldrich Company Ltd., UK	89640
Triethanolamine	Sigma-Aldrich Company Ltd., UK	T5-830-0
Tris base	Fisher Scientific, UK	BP152-1
Tris-borate-EDTA (TBE) buffer	Pomega Inc., UK	V4251
Tri-sodium citrate	Fisher Scientific, UK	S/3320/60
Triton X-100	Sigma-Aldrich Company Ltd., UK	T8787
Xylene	Genta Medical, UK	EC 215-535-7

Yeast tRNA	Sigma-Aldrich Company Ltd., UK	R8759

2.1.2 Kits and packs

Item	Company	Catalogue number
Agilent Brilliant Ultrafast SYBRgreen Mix	Agilent, UK	600886
DIG RNA Labelling Mix	Roche Diagnostics Ltd., UK	11277073910
DIG Wash and Block Buffer Pack	Roche Diagnostics Ltd., UK	115857621
Direct-zol RNA Miniprep	Qiagen, UK	R2050
Fast Start Taq Polymerase dNTP Pack	Roche Diagnostics Ltd., UK	47383571
High Capacity Reverse Transcription Kit	Fisher Scientific, UK	4368814

Qiagen Plasmid Midi Prep Kit	Qiagen, UK	12145
Qia Quick Nucleotide Removal Kit	Qiagen, UK	28304
SP6/T7 transcription Kit	Roche Diagnostics Ltd., UK	109996441
Terminal Transferase	Roche Diagnostics Ltd., UK	3289869103
Testosterone ELISA Kit	Enzo Life Sciences, UK	ADI-901-65
Vectastain Elite IgG anti- rabbit ABC kit	Vectastain, UK	PK-6101

2.1.3 Restriction enzymes and buffers

Item	Company	Catalogue number
Bovine Serum Albumin	Pomega Inc., UK	R396D
Buffer D	Pomega Inc., UK	R004A
Buffer S	Pomega Inc., UK	R002A

10x Multi buffer	Pomega Inc., UK	R99A
SPeI restriction enzyme	Pomega Inc., UK	R659A
Xbai restriction enzyme	Pomega Inc., UK	R618A

2.1.4 Steroids

Item	Company	Catalogue number
Beta-estradiol 3-benzoate	Sigma-Aldrich Company Ltd., UK	E8515
Diethylstilbestrol	Sigma-Aldrich Company Ltd., UK	D4628
Progesterone	Sigma-Aldrich Company Ltd., UK	P0130
Testosterone propionate	Sigma-Aldrich Company Ltd., UK	T1875

2.1.5 Primary antibodies for immunohistochemistry

Item	Company	Catalogue number
Oxytocin primary antibody	Immunostar, Inc., USA	20068
K 25 C-fos primary antibody	Santa Cruz Biotechnology, Inc., USA	sc-253
GnIH primary antibody	Gift from Prof George Bentley, University of California, Berkeley, USA	

2.1.6 Radioactive Isotopes

Item	Company	Catalogue number
^{35}S dATP	Perkin Elmer, UK	NEG03425 UC
^{125}I Ornithine Vasotocin Analog	Perkin Elmer, UK	NEX25401 0UC

2.2 Methods

This section contains information on the experimental animals, general laboratory techniques and data analysis methods used in following chapters. Additional details are given when necessary in relevant chapters.

2.2.1 Experimental birds

Details of the specific types of birds used can be found in respective chapters.

All birds used in this project - J-line chickens (a line produced and maintained at the Roslin Institute Poultry Unit, used in experiments described in Chapter 3), White Legnorn/Silkie cross (the F1 generation of a cross between male Silkie and female White Leghorn birds, used in experiments described in chapters 4, 5 and 6) and Japanese quail (used in experiments described in Chapter 7) were sourced from colonies created and maintained at the Roslin Institute poultry facility. All birds were treated in accordance to the UK Home Office Guidelines. Birds were given visual health checks and weighed at appropriate times during experiments. Only birds of healthy weights were used. All birds were kept in floor pens $2m^2$ covered in wood shavings with varying numbers of birds per pen but not exceeding a maximum of 8 birds with weights > 2.4kg. Temperature was kept between 18 and 23°C and all birds had access to food and water *ad libitum* at all times. Standard lighting conditions were 14 hours of light and 10 hours of darkness, lights on at 6am. When necessary to promote incubation, adult females were kept in floor pens

equipped with nest boxes (30x50x28 cm) containing either fertile or non-fertile chicken eggs.

All adults and juveniles used in experiments were killed by an overdose of anaesthetic (pentobarbital administered through injection into the wing vein at a dose of 50mg/kg body weight). All chicks were killed by cervical dislocation.

2.2.2 General introduction to polymerase chain reaction (PCR), sex determination for young chickens and gel electrophoresis

2.2.2.1 General introduction

Details of the specific protocols used can be found in respective chapters.

PCR is a technique used to amplify a fragment of a DNA sequence, resulting in a very high number of copies which allow for easier detection of that sequence. It is widely used in both clinical practice and research. PCR involves exposing the reagents repeatedly to high and low temperatures in a specific sequence thus allowing the temperature-dependent melting and replication of DNA to proceed many times in succession. The method requires a DNA polymerase enzyme (usually Taq polymerase which is heat-stable), free nucleotides and primers - short DNA sequences complementary to the desired DNA sequence which serve as starting points for replicating the fragment. Choosing primers which are highly specific to the fragment of interest is an important consideration as the wrong fragment may be amplified otherwise. Once a new fragment is created it begins serving as atemplate along with the original template and the number of copies begins growing exponentially.

2.2.2.2 Sex determination

Details of the sex determination procedure can be found in the respective chapter.

As the sex of juvenile chickens is not apparent, an established PCR sexing method was used (Clinton et al., 2001). Primers W3 and W5 amplify a 415 bp (base pair) product of the XhoI repeat sequence present only in females while primers R1 and R2 amplify a 256 bp product of the 18S ribosomal gene (primer sequences further below). When a PCR reaction using DNA from a specific chick (in this case DNA purified from blood) is set up to amplify these two fragments, then run on an agarose gel through gel electrophoresis and visualised under a UV light, two bands will be visible if the chick is female and only one band if the chick is male. Gel electrophoresis is a technique in which negatively charged molecules (in this case DNA) are moved through a matrix (most often an agarose gel) by an electric field. Smaller molecules move faster than larger ones, allowing the separation of nucleotide fragments by size. A marker or 'ladder' consisting of fragments of known size is usually run on the same gel to help determine the size of an unknown fragment.

2.2.3 Collection of chicken brains

Details of other tissue and blood collection methods can be found in respective chapters.

The entire brains and, where appropriate, the pituitaries (both lobes together) of all birds were dissected out, immediately frozen on dry ice and stored at -70°C. Pituitaries were weighed prior to freezing. Brains were sectioned coronally on a cryostat at 15µm, mounted on glass microscope slides and kept at -70°C with a silica gel bag to remove moisture until use. Brains were sectioned from the front of the brain behind the olfactory bulb up to the most dorsal part of the PVN.

Sections were mounted on a marker slide for every four experimental slides and counterstained with Toluidine blue to assist in the selection of experimental slides containing the desired region.

2.2.4 Design of oligonucleotide probes for *in situ* hybridisation (ISH)

The oligonucleotide sequences of both of the probes used in experiments covered in Chapter 3 and 4 can be found in the Chapter 3 Materials and Methods section.

Oligonucleotide probes are synthetic polynucleotides synthesised artificially to match any desired sequence. Oligonucleotide probes have a number of advantages over other probes used for ISH (RNA and cDNA probes). They are cheap and commercially available and can be specifically designed to

target the most variable sequence of a gene. They consist of only a single strand thus avoiding possible renaturation which is an issue with double-stranded probes. They are small in size (35-50 nucleotides) which permits them to easily enter cells. They are also resistant to RNAses (enzymes which cut RNA) which can be a major problem when RNA probes are used. As they can be designed by the user, it is also possible to control their G/C content which in turn influences the hybridisation conditions necessary for the procedure.

Oligonucleotide hybridisation probes complementary to the desired mRNA were designed with the Primer3 web tool (http://primer3.ut.ee/) from the chicken vasotocin protein-coding nucleotide sequence and the chicken mesotocin protein-coding nucleotide sequence. All sequences were found on the NCBI online database and probes were designed to span an exon. BLASTn (NCBI) was used to confirm that the region used to design the oligonucleotides matched only the desired peptide. The best sequences in terms of low secondary structure and hairpin loop formation were chosen and tested by running a test ISH.

2.2.5 General introduction to immunohistochemistry

Details of the specific protocols used can be found in respective chapters.

Immunohistochemistry (IHC) is a procedure used to visualise proteins (antigens) in cells and study their distributions in tissues. IHC relies on the ability of antibodies to bind specific antigens. In order for the protein to be visualised usually a reaction which produces colour must take place. This is most often achieved by conjugating the antibody to an enzyme (peroxidase is commonly used) which produces colour in the presence of substrate.

In the procedures described here a polyclonal primary antibody (polyclonal antibodies have higher affinity and amplify targets with lower expression as asingle target will bind more than one antibody molecule) was used together with a biotinylated secondary antibody. Avidin and biotin bind together with very strong affinity, forming an avidin biotin complex. This interaction is utilised to further amplify the signal. The secondary antibody bound to the primary antibody and was followed by the enzyme horseradish peroxidase (HRP) which produces the colour reaction in the presence of the chromagen substrate DAB (3,3-diaminobenzidine) and hydrogen peroxide and which was in turn attached to an ABC (avidin-biotin complex), ultimately resulting in a large number of peroxidase molecules being bound to the protein of interest, making it easy to detect. In the procedures described in following chapters, the pH of 1xPBS and 1xPBS-T was always between 7.35 and 7.42. '1x' corresponds to a 0.1M solution.

2.2.6 Radioactive *in situ* hybridisation

In situ hybridization histochemistry (ISH) involves using a labelled nucleotide probe which hybridizes to a specific nucleotide sequence, making it detectable. This technique was used to localise and quantify mRNA in the chicken brain. There are different types of probes which can be used including DNA, RNA (riboprobes) and oligonucleotide probes. As already described above, for the radioactive ISH oligonucleotide probes were used as they are cheap, specific and robust and can easily enter cells. A ^{35}S radioisotope was used to label the probe in this case. Autoradiographic emulsion is used to visualise the signal. It contains silver halide crystals which become activated by the radioactivity emitted by the ligand. Following development, the activated crystals become black silver grains and can be observed and quantified under the microscope. The procedure used in this

thesis has been previously described (Krause et al., 2015) and used by the Meddle lab.

2.2.6.1 Probe labelling

The unlabelled oligonucleotide probes for mesotocin and vasotocin designed earlier were diluted in sterile water to stock solutions with concentration 100pmol/µl. The stock solution was stored at 10µl aliquots at -20°C until required.

On the day the probes were to be labelled the stock solutions were further diluted with sterile water to a working concentration of 10pmol/µl.

Probes were labelled in a 50µl reaction mix containing 26.5µl sterile ddH_2O, 10µl 5x terminal deoxynucleotidyl transferase (TdT) Tailing Buffer (Thermo Fisher Scientific), 5µl 25mM Cobalt chloride ($CoCl_2$) (Thermo Fisher Scientific), 5µl ^{35}S dATP, 2µl probe (working concentration 10pmol/µl) and 1.5µl TdT enzyme (Thermo Fisher Scientific). This was mixed well by repeated pipetting in a sterile Eppendorf. The Eppendorf was placed in a pre-heated water bath at 37°C for 1.5 hours, after which it was cooled briefly on ice to halt the reaction.

A QIA Quick Nucleotide Removal Kit (Qiagen) was used for purifying the probe following the manufacturer's protocol. 10 volumes of buffer PN were added to 1 volume reaction sample and this was transferred to a spin column placed in a 2ml collection tube. The column was centrifuged for 1min at 4000 x g. The flow through was discarded and the spin column was placed in a clean collection tube. 500µl of PE buffer containing 100% ethanol were added to the column and the column was again centrifuged for 1 min at 4000 x g. The flow through was discarded and the same was repeated one more time using the same collection tube. After discarding the second flow through

the same collection tube was used to centrifuge the sample for 1 min at top speed. The spin column was placed into a clean collection tube and 50µl EB buffer were pipetted onto the resin filter at the centre of the column. The column was left to stand at room temperature for 1 minute and then centrifuged for 1 min at top speed. The flow through (the labelled probe) was decanted into a sterile centrifuge tube. The activity of the probe was counted with a Hidex beta-counter (Hidex Oy.) by placing 1µl of the probe in 3.5ml scintillation fluid in a scintillation vial. The labelled probe was stored at -20°C.

2.2.6.2 Pre-hybridisation

Pre-selected slides in a sealed slide box were removed from the -70°C freezer and left at room temperature for 2 hours. Slides were then fixed in 4% paraformaldehyde in 1xPBS for 10 minutes and then given two 5-min washes in 1xPBS. Slides were then incubated for 10 min in Triethanolamine/acetic anhydride (TEA/AA) solution where AA was added and stirred briefly directly before immersing the slides. Slides were rinsed briefly in ddH_2O and put through 70%, 95% and 100% ethanol, then 100% chloroform and 100% and 95% ethanol again for 3 min in each before being left to air dry at room temperature for 30 minutes.

2.2.6.3 Hybridisation

A hybridization solution was prepared including the labelled probe (amount calculated depending on activity, aiming for 100 000 cpm per section, approx. 4 sections per slide), hybridization buffer and dithiothreitol (DTT) at 20µl/ml. 25µl of the hybridization solution were pipetted onto each section on the

slides. Nescofilm was used to coverslip the slides and ensure the even distribution of the solution across the tissue. The slides were placed in a humid hybridisation chamber and incubated overnight in a hybridisation oven at 37°C.

After hybridisation was complete, each slide was given three brief rinses in saline-sodium citrate buffer (SSC) used to control the stringency during washing. 1xSSC (Diluted from 20x stock containing 175.3g/l sodium chloride (Fisher) and 88.2g/l tri-sodium citrate (Sigma)) was used. The rinses were performed at room temperature and during them the Nescofilm coverslip was removed. Slides were then given four 15-minute washes in a water bath in 1xSSC at a temperature 20 degrees below the melting temperature of the probe. This was followed by two thirty-minute washes in 1xSSC at room temperature. Slides were rinsed briefly in ddH_2O (double-distilled water) and allowed to air dry overnight.

2.2.6.4 Dipping in emulsion

The following day Ilford K5 Gel Emulsion diluted to 75% in sterile water was removed from storage at 4°C and left at room temperature in a sealed box for 2 hours before being placed in a water bath under safe light conditions at 42°C for another two hours. Slides were then dipped in emulsion and left to overnight under safe light to dry in light tight boxes (2 days) before being transferred to a sealed light tight box with a silica gel bag and moved to 4°C to be exposed for a further 17-21 days.

2.2.6.5 Visualisation

Once slides had been exposed for the appropriate amount of time, slide boxes were removed from 4°C and left on bench for 60 min to come to RT. Fixative and Phenysol liquid developer (Ilford) were diluted 1 in 5 in ddH_2O before use under safe light conditions. Slides were put in slide racks and immersed once in developer, twice in fixative and three times in ddH_2O for 5 min each before being counterstained in autostainer with hematoxylin “Z”, dehydrated through an alcohol series (70% ethanol, 95% ethanol, 50/50 95% ethanol and xylene and pure xylene) and coverslipped.

2.2.7 General introduction to digoxigenin-labelled *in situ* hybridisation

Details of the specific protocol used can be found in the respective chapter.

While using a radioactive isotope is one of the most robust quantifiable methods of labelling ISH probes, quicker and cheaper methods which do not involve the use of radioactivity also exist. One of these methods involves the use of Digoxigenin (DIG) - a steroid produced by plants and often used as an immuno-tag because it is easily detectable by antibodies labelled with dyes and can thus be visualised. When used in ISH DIG is conjugated to an RNA nucleotide which is then incorporated into an RNA probe (riboprobe). Riboprobes are larger, more expensive and difficult to produce and highly susceptible to degradation by RNases but they are suitable for use in this type of non-radioactive ISH. This procedure was used to detect GnIH mRNA in the chicken brain as it was time- and cost-effective and the robustness of the procedure had already been demonstrated in quail (Satake et al., 2001).

2.2.8 General introduction to receptor-binding autoradiography

Details of this procedure can be found in the relevant chapter.

Receptor-binding autoradiography was used to determine the localisation and intensity of mesotocin receptor-binding in the chick brain. In this procedure a radioactively-labelled ligand specific to the receptor of interest is introduced to the tissues. The bound radioactive ligand can then be visualised by apposing it to autoradiography film coated in autoradiographic emulsion. The process of visualisation of film is similar to that for development of ISH. The intensity of the black colour can be used to determine the level of binding which has occurred. The procedure used in this thesis has been previously used by the Meddle lab and has been described by Insel and Shapiro (Insel and Shapiro, 1992).

2.2.9 Image capture and analysis

2.2.9.1 Image analysis for radioactive ISH

Slides were coded so that the person analysing them was blinded to the identity of each birds and thus avoid bias. When appropriate, cells were counted manually under the microscope at an appropriate magnification. When image analysis software (Image J) was to be used, photographs of the regions of interest (ROI) on both the left and right side of the brain were taken at the appropriate magnification on a Nikon E600 or Nikon E400 (Nikon

Co., Ltd) microscope, using ZEISS camera and the ZEN software (ZEISS Microscopy). Within a set of images to be compared all settings were kept constant throughout the capturing process. The mRNA or protein signal density in the ROI in % area covered by signal (equivalent to mm^2/mm^2) was measured with the Image J image-processing software (Image J) by applying a threshold which only counted pixels above a certain intensity and drawing manually around the region of interest. The same threshold was used for all images which were being compared. A background measurement was also taken from an adjacent area for each image and subsequently subtracted from the ROI measurement to eliminate the effect of different background levels on the accuracy of the final value. The measurement for the background, like that for the area of interest was in % area covered by signal (equivalent to mm^2/mm^2). Measurements were taken on each side of the brain (hemi-section) and measurements from both sides were pooled together and an average value was calculated per bird. The number of measurements varied between 4 (4 hemi-sections measured) and 12 (12 hemi-sections measured) depending on the size of the region and the availability of undamaged sections which could be analysed for a given area. Details on the number of measurements can be found in relevant chapters. Brain areas to be measured were identified with the help of the Stereotaxic Atlas of the Brain of the Chick (Kuenzel and Masson, 1988).

2.2.9.2 Image analysis for DIG ISH, c-fos and double labelling IHC on free-floating sections and IHC for GnIH

Slides were coded to avoid bias as above and labelled cells or nuclei were counted manually under the microscope. For double-labelled cells, each cell was examined at different focus depths to confirm the presence of a labelled nucleus within the labelled cytoplasm.

2.2.10 General introduction to quantitative polymerase chain reaction (qPCR)

Details of the specific protocol used can be found in the respective chapter.

Quantitative polymerase chain reaction (qPCR) also known as real-time PCR can be used to quantify the expression of a gene of interest in a tissue sample. In qPCR the amplification of the targeted DNA fragment (reverse-transcribed from the RNA in the experimental sample) is monitored and recorded in real time with the help of a reporter (in this case a fluorescent dye which binds double-stranded DNA). The level of amplification is proportional to the abundance of the target sequence in the original sample and the concentration of the target can be calculated from a standard curve constructed from samples of known concentration and run in the same reaction as the unknown samples. The procedure used in this thesis has been previously described (Reid et al., 2017).

2.2.11 General introduction to Liquid Chromatography Mass Spectrometry (LC-MS)

Details of the specific protocol used can be found in the respective chapter.

This assay was developed and optimised at The Roslin Institute by Dr Andrew Gill who also performed some of the sample preparation and operated all equipment. This procedure was used to quantify the amounts of dopamine, serotonin, adrenaline and noradrenaline in the chicken Raphe

nucleus. High-performance liquid chromatography (HPLC) is a procedure which allows the user to quantify a number of compounds in a mixture by passing the sample mixture through a column containing adsorbent material. As different chemical compounds pass through the column at different speeds, this allows for their separation. A detector in the HPLC instrument emits a signal corresponding to the quantity of each compound as it comes out of the column. In our case this was based on mass spectrometry (MS) which sorts chemical compounds based on their mass-to-charge ratio. This allows for the amount of each chemical of interest to be quantified. Standards of known concentrations are used for this purpose. The procedure used in this thesis has been previously described (Ritchie et al., 2002).

2.2.13 General introduction to Enzyme-linked Immunosorbent Assay (ELISA)

In this project ELISA was used to measure plasma concentrations of testosterone in laying, incubating and chick-rearing hens. Details of the specific protocol used can be found in the respective chapter.

An enzyme-linked immunosorbent assay (ELISA) is a procedure used widely in research and diagnostic medicine which utilises an antibody-antigen interaction combined with a colour reaction to detect and quantify specific antigens in a sample. It involves immobilisation of the antigen of interest to a surface and then treatment with a specific antibody combined with an enzyme which produces a colour reaction. Once the enzyme substrate is added the amount of antigen in the sample can be quantified with the help of a spectrophotometer based on the intensity of the colour produced.

2.2.14 Statistical analysis

More details on the specific statistical tests used can be found in the respective chapters.

Statistical software used for all tests and statistics was Sigmaplot (version 14.0, Sigmaplot Software Inc.). The Shapiro-Wilk normality test was used to confirm a normal distribution.

In most cases, One-way ANOVA using treatment as the independent variable (or Kruskal-Wallis one-way ANOVA on ranks when data were not normally distributed) and Tukey's or Dunn's post-hoc test respectively were performed on all data. $P<0.05$ was considered statistically significant in all cases. Student's t-test or Welch's test were also used when relevant. The tests used are noted in relevant chapters.

Chapter 3 Effects of Gonadal Steroids on the Mesotocin and Vasotocin System in the Brain of the Juvenile Domestic Hen

Abstract

Magnocellular and parvocellular neurones in the paraventricular nucleus (PVN) and bed nucleus of the stria terminalis (BnST) of birds are responsible for producing the neuropeptides mesotocin and vasotocin. Previous studies indicate that embryonic differentiation of the mesotocin and vasotocin systems in the chicken is affected by gonadal steroids. In female quail, vasotocin neurones in the medial division of the BnST (BnSTm) and the PVN are sensitive to both testosterone and estradiol. However, the effects of treatment with gonadal steroid hormones on vasotocin neurones in the brain of the female chicken post-hatch or on mesotocin neurones in the brain of any bird species post-hatch are not known. Studying mesotocin and vasotocin's interactions with other peptides is crucial for understanding the mechanism of their action as well as being able to control for various factors during experiments. In the study of maternal behaviour, it is important to know whether any changes in the expression of these peptides in the brain are triggered directly by behavioural and environmental factors or are triggered instead by gonadal steroids. Studies so far have focused mainly on the BnSTm while the lateral BnST has not been studied. As the BnSTl is involved in the regulation of the stress response and stress responses are influenced by both gonadal steroids and nonapeptides, it is beneficial to examine how they interact in this brain region. The following experiments aimed to determine whether a single injection of one of three sex steroids - testosterone propionate, estradiol benzoate or progesterone - administered after a period of priming with the synthetic estrogen diethylstilbestrol (DES), had any effect on mesotocin or vasotocin mRNA expression in the PVN or

BnSTl, on mesotocin immunoreactivity in the PVN or on mesotocin receptor binding in the lateral septum (LS). Treatment with testosterone increased mesotocin mRNA expression in both the PVN and BnSTl while none of the other treatments had a significant effect on mesotocin mRNA expression. There was no difference between groups in the density of meotocin-immunoreactive neurons in the PVN or mesotocin receptor binding in the LS. Testosterone and estradiol both increased vasotocin mRNA expression in the BnSTl to similar extents but none of the treatments had any effect on the vasotocin mRNA expression in the PVN. These findings demonstrate for the first time that acute treatment with gonadal steroids outside of the embryonic period can upregulate the mesotocin and vasotocin systems in the brain of the domestic hen and that the BnSTl is one of the brain regions sensitive to these steroids.

3.1 Introduction

3.1.1 Overview of sex steroids and their actions

Testosterone, progesterone and estrogen/estradiol are steroid hormones often referred to as ‘sex steroids’ which are secreted by the gonads, interact with vertebrate androgen, progesterone and estrogen receptors respectively and are involved in the control of reproduction (Andrew, 1963; Beach and Inman, 1965; Davidson, 1966; Hart, 1967; Hillsman et al., 2007; Moore and Zoeller, 1979; Panzica et al., 1996). While sex steroids are most known for the regulation of sexual behaviours, they can also strongly influence the hypothalamus-pituitary-adrenal (HPA) axis to affect stress responses. Stress responses have been shown to be sexually dimorphic in studies in rats and birds and treatment of gonadectomised animals with estrogen increased

while treatment with testosterone decreased the stress response (Critchlow et al., 1963; Dickens and Bentley, 2014;; Handa et al., 2011; Klukowski et al., 1997; Larkin et al., 2010; Nagra et al., 1965; Seale et al., 2005a; Seale et al., 2005b; Zysling et al., 2006). This is not a surprise as studies have shown that the stress system is tightly connected to reproduction and the stress response is often attenuated during the parental stage of the reproductive cycle – for example, it is lower in lactating compared to non-lactating rats, as well as in parental compared to non-parental Lapland longspurs experiencing bad weather conditions (Douglas et al., 2007; Krause et al., 2016). In addition, sex steroids may influence other behavioural and metabolic responses in mammals through their interactions with oxytocin (Bale and Dorsa, 1995; Caldwell et al., 1989; Caldwell et al., 1988; Chibbar et al., 1990; De Kloet et al., 1985; Goudsmit et al., 1988; Johnson et al., 1989; Panzica et al., 1996; Patisaul et al., 2003) and vasopressin (De Vries et al., 1983; Grozhik et al., 2014; Seth et al., 2004), and in birds through interactions with vasopressin's avian orthologue vasotocin (de Vries et al., 1984; Grozhik et al., 2014; Kimura et al., 1999; Plumari et al., 2004; Seth et al., 2004; van Leeuwen et al., 1985; Viglietti-Panzica et al., 1994; Voorhuis et al., 1988b). As noted before, nonapeptides are themselves involved in stress but also in the control of homeostasis (vasopressin, as its name suggests, is a vasopressor, while oxytocin can act as both a vasopressor and vasodepressor), as well as in the facilitation of social behaviours including, at least in the case of oxytocin and vasopressin, the promotion of maternal behaviour (Bayerl et al., 2016; Bosch and Neumann, 2008, Bosch and Neumann, 2010; Bosch et al., 2010; Bottje et al., 1989; Chaturvedi et al., 1994; Fahrbach et al., 1984; Goodson and Evans, 2004; Hewlett et al., 2014; Kaufmann et al., 2000; Kelly and Goodson, 2014; Marrian and Newton, 1935; Parkes, 1930; Pedersen et al., 1982; Petty et al., 1985; Selvam et al., 2013; Sharma and Chaturvedi, 2011; Sharma et al., 2009; Wang et al., 1989). The effects of sex steroids on mesotocin, the avian orthologue of oxytocin and a likely regulator of maternal behaviour in birds (Thayananuphat et al., 2011),

have not been established. Sex steroid concentrations are generally high in the plasma of birds during the reproductive period and decline with incubation (Gratto-Trevor et al., 1990; Potter and Cockrem, 1992), but as they have been shown to upregulate oxytocin in mammals (Bale and Dorsa, 1995; Caldwell et al., 1989; Caldwell et al., 1988; Chibbar et al., 1990; De Kloet et al., 1985; Goudsmit et al., 1988; Johnson et al., 1989; Panzica et al., 1996; Patisaul et al., 2003), it is reasonable to expect that they may have similar effects in birds. If this is the case, then further studies would be necessary to establish why, if high levels of sex steroids and maternal behaviour are both connected to elevated mesotocin in the same brain areas, they occur at different times in the reproductive cycle and the circulating sex steroids in laying birds do not induce maternal behaviour.
Brain areas where nonapeptide mRNA, protein or receptor binding have been shown to be increased by testosterone and/or estrogen, include the PVN, MPOA/POM, LS, BnSTm and ventromedial hypothalamus (VMH) but, to the author's knowledge, the BnSTl had not been previously examined in this context.

3.1.2 Aim and hypothesis

In previous studies, sex steroids have been shown to be capable of upregulating nonapetide systems in areas of the brain behaviour network of both mammals (oxytocin and vasopressin) and birds (vasotocin). Based on the existing literature, it was predicted that these steroids would also upregulate the mesotocin and vasotocin systems in the brain of the female chicken. The experiment described in this chapter aimed to address the gap in the literature concerning the effects of sex steroids on avian mesotocin in its main site of synthesis - the PVN - as well as mesotocin immunoreactivity in the same area and receptor binding to the avian mesotocin receptor (OTR-

like, also known as VT3) in the LS, which is one of mesotocin's major sites of action and is involved in social and sexual behaviours, as well as the stress response. While no data for mesotocin from other avian species was available, the existing data on the effects of sex steroids on the oxytocin system in the brain, as well as on vasopressin and vasotocin, which are also closely related to mesotocin, suggested that they were likely to promote mesotocin mRNA expression, protein synthesis and receptor binding in the examined areas.

To the author's knowledge, the acute effects of gonadal steroid hormones on the vasotocin system in the female chicken had not been examined prior to this study. However, once again, data exist to suggest that they upregulate the vasotocin system (including the PVN and the BnSTm) in the brains of other avian species, including both songbirds and quail, so similar effects were expected in the chicken. Unfortunately, immunoreactivity and receptor binding for vasotocin were not measured as previous attempts by the Meddle lab to obtain an adequate radioactive ligand for receptor binding autoradiography, as well as the testing throughout this project of several antibodies and protocols for immunohistochemistry for this peptide, were unsuccessful.

An additional area examined for mRNA expression for both peptides was the BnSTl, which has been shown to be involved in social interactions and stress. This area had not been focussed on in previous studies with sex steroids. However, data were available for the medial section of the same nucleus and, as the BnSTl and BnSTm are closely related and both express mesotocin and vasotocin, it was predicted that the effects of sex steroids on the BnSTl would be similar to those on the BnSTm where nonapeptide expression was shown to be upregulated by them.

As noted before, juvenile birds were used in this experiment as a cheaper alternative to ovariectomised adult individuals, as they do not produce their own sex steroids at this age. It was determined that using adults and

performing castration would have been too expensive, difficult and time-consuming considering the overall aims and timeline of this PhD project. While it is possible that there may be differences between juveniles and adults, the results presented in this chapter corresponded well to results observed in adult quail. Avoiding surgery also had the added benefit of reducing the negative impact on the welfare of the animals. While repeating the study in adult chickens in the future to confirm results may be beneficial, it was determined that data from this study would still be valuable, especially as some of these hormones and brain areas were being examined for the first time in this context.

Hypothesis:

Based on the existing literature in both mammals and birds cited previously, it was hypothesised that acute administration of sex steroids would induce mRNA expression in the PVN and BnSTl, immunoreactivity in the PVN and receptor binding in the LS of the female chicken for both mesotocin and vasotocin (although receptor binding and immunoreactivity for vasotocin could not be tested).

3.2 Materials and methods

Details of the materials used in this chapter as well as the in situ *hybridisation protocol used can be found in Chapter 2. What follows is a description of the procedures specific to this chapter.*

3.2.1 Animals and housing

J-line chicks (*Gallus domesticus*) hatched from the Roslin Institute breeding colony were housed from the day of hatching in $2m^2$ floor pens equipped with heat lamps for the first 3 weeks post-hatch in a quiet experimental room under standard lighting conditions (14L:10D, lights on at 6am). Food (starter diet) and water were provided *ad libitum*. The birds used in the experiment were from a total of eight hatches hatched on different days and all animals were treated in accordance to the Home Office Guidelines. Birds were randomly assigned to groups.

3.2.2 Determination of the sex of juvenile chicks by polymerase chain reaction (PCR) and gel electrophoresis

A general introduction to this procedure can be found in Chapter 2.

Blood samples for PCR sexing (5µl for each bird) were collected through punching a small hole in the wing vein of each chick before undergoing DNA extraction as described below. The birds were one week old at the time of sample collection. Female chicks were used in this experiment.

3.2.2.1 DNA extraction

5µl of blood were collected from each chick and mixed with 400µl DNAzol. DNAzol is a reagent used to isolate genomic DNA from a blood sample. The samples were spun in a centrifuge for 4 minutes at top speed at room temperature (RT) and the supernatant was carefully removed. 300µl isopropanol were added to each tube and the samples were spun again under the same conditions. The supernatant was once again removed, and the samples were given two spinning washes in 300µl 100% ethanol at top speed for two minutes. After this they were left to air dry at room temperature for 30 minutes before being resuspended in 400µl miliQ water (from Q-POD, Elix) by briefly vortexing. This resulted in purified genomic DNA (gDNA).

3.2.2.2 PCR and gel electrophoresis

The primers used in the PCR reaction were W3 with sequence 5'-GAA ATG AAT TAT TTT CTG GCG AC-3' and W5 with sequence 5'-CCC AAA TAT AAC ACG CTT CAC T-3' the two of which amplified a female-specific chromosome and R1 with sequence 5'-AGC TCT TTC TCG ATT CCG TG-3' and R2 with sequence 5'-GGG TAG ACA CAA GCT GAG CC-3' which amplified a ribosomal gene sequence present in both sexes.

Primers were all diluted in sterile ddH_2O to 10pm/l. 0.4µl of primers W3 and W5 each and 0.5µl of primers R1 and R2 were added to 1.5µl 10xNTP and 1.5µl 10xBuffer along with 1.2µl of 2mM Mg_{2+}, 3.0µl of 16.5% (w/v) Bromophenol Blue and Sucrose, 0.75µl Dimethyl sulphoxide (DMSO), 0.075µl Taq Polymerase, 3.175µl ddH_2O and 2.0µl purified gDNA. Thermal conditions were as follows: 94°C for 2 minutes, 94°C for 10 seconds, 54°C for 15 seconds, 72°C 20 seconds, run for 30 cycles and 72°C for 5 minutes.

The PCR product was then loaded onto a 1% agarose (in Tris-acetate EDTA a.k.a TAE buffer) gel for analysis along with a 100bp DNA ladder. 2µl SYBR Safe (Invitrogen) per 100mls of agarose were added. SYBRSafe is a safe alternative to ethidium bromide which was previously used. It can be detected under ultraviolet (UV) light, it binds to DNA and enables it to be visualised. The gel was run at 100V for 30 minutes and visualised and photographed using the UV Light/Geldoc system.

3.2.3 Gonadal steroids

It has been demonstrated in previous studies that the facilitation of gene expression by sex steroids in the chicken can be enhanced through priming with the synthetic estrogen diethylstilbestrol (DES). Pre-treatment with 0.5mg DES per bird per day was demonstrated to amplify the inducing effects of a single injection of sex steroids on avidin mRNA in the chick oviduct (Kunnas et al., 1992). This Kunnas *et al* study was used as the basis for the protocol for the present experiment, using DES to amplify any effects the injected steroids might have on the genes of interest in order to ensure that such effects would be easily observable. The steroid doses used in this experiment were based on those used in the above study to induce avidin expression (Kunnas et al., 1992). The dose used for testosterone was also very similar to the dose used by Aste *et al* in their study in quail in which a dose of 22µg testosterone per gram body weight promoted vasotocin mRNA expression in the BnSTm of females (Aste et al., 2013).

3.2.3.1 Steroid preparation

Steroid hormones were dissolved or suspended in corn oil on a heating platform with a stirrer. Diethylstilbestrol (DES) was dissolved at 1g/ml, testosterone propionate (referred to as testosterone further down) and progesterone were dissolved at 20mg/ml and β-estradiol 3-benzoate (referred to as estradiol further down) was suspended at 10mg/ml. Suspension was prepared right before use and was stable at the time of injection, ensuring that the correct dose was delivered.

3.2.3.2 Estrogen priming

Chicks no younger than 13 and no older than 15 days with weight of at least 70g were primed by injecting 1g of the artificial estrogen diethylstilbestrol (DES) in 100µl corn oil (50µl in each side) into the pectoral muscle. The injections were repeated on days 3 and 5 from the first injection. Sixteen days were allowed for withdrawal. On the first and second day after this period, the birds received secondary priming with 0.5g DES in 50µl corn oil into the pectoral muscle. Chicks were weighed before each injection.

3.2.3.3 Steroid injection

24 hours after the last priming injection the birds received a single injection into the pectoral muscle of either vehicle (corn oil, 1ml/kg body weight), estradiol (10mg/kg body weight), progesterone (20mg/kg body weight) or

testosterone (20mg/kg body weight) dissolved or suspended as described above.

3.2.4 Tissue collection and sectioning

24 hours after steroid or vehicle injection the birds were killed with an overdose of anaesthetic (for details, see Chapter 2). Brains and the gonads were collected immediately after death, frozen on dry ice and stored at -70˚C. Coronal brain sections were cut on a cryostat at 15µm, mounted on polysine slides and stored at -70˚C. As gonadal steroid hormones are known to influence the size of the oviduct, the oviducts of chicks were collected and weighed as confirmation that the injected hormones were present in the system and having an effect.

3.2.5 Immunohistochemistry for mesotocin on slides

A general introduction to this procedure can be found in Chapter 2. What follows is the protocol used for the experiment described in this chapter. The Vectastain Elite IgG anti-rabbit ABC Kit was used.

Pre-selected slides were removed from the -70° freezer and fixed in 4% Paraformaldehyde (PFA) for 30 min. before being given three five-minute washes in 1xPBS (diluted from 10x stock containing 1.4M NaCl, 0.027M KCl, 0.1M Na_2HPO_4 and 0.018M KH_2PO_4). The purpose of fixation is to preserve cell structure while immobilising the target antigens. Endogenous peroxidases were quenched for 15 min with H_2O_2 in a solution containing

20% methanol, 0.02% Triton, 5% H_2O_2, 50% 2xPBS and 24.08% H_2O. This step is necessary in order to reduce nonspecific staining resulting from the activity of peroxidases present in the tissue. Saturation with H_2O_2 effectively inactivates these enzymes. The solution was stirred for 1 min before use. Slides were washed for 15 min in 0.2% Triton in 1xPBS (1xPBS-T) and then incubated for 60 min in blocking solution consisting of 10% Normal Goat Serum (NGS) in PBS-T. This blocking step is performed with normal serum from the species in which the secondary antibody is raised, in this case goat. This serum contains antibodies which bind to non-specific sites in the tissue, thus preventing the conjugated antibodies from binding non-specifically and producing high background noise. Triton is a detergent which makes the tissue more permeable.

A hydrophobic immuno pen was used to draw around the tissue and slides were transferred to humidifying rack. 400µl primary oxytocin antibody (raised in rabbit) at concentration 1:1000 were applied per slide. Slides were incubated on bench at RT for 60 min and then transferred to -4°C for overnight incubation.

After the overnight incubation slides were washed three times for 15 min in 1xPBS-T. Hydrophobic immuno pen was reapplied and 400µl biotinylated anti-rabbit immunoglobulin G (IgG) antibody diluted to a concentration of 1:100 in 1xPBS-T with 3% NGS were applied to each slide. Slides were incubated at RT on bench for 60 min before once again transferring to -4°C for overnight incubation.

After this incubation slides were again washed three times for 15 minutes in 1xPBS-T. Hydrophobic immuno pen was reapplied and 400µl per slide of ABC solution prepared 30 min before use with 10µl/ml of reagent A and B each in 1xPBS-T (Vectastain Elite Rabbit IgG ABC Kit) were applied. Slides were incubated at room temperature for 60 min. This was followed by three 10-minute washes in 1xPBS-T and a 5-min rinse in 1xPBS. Slides were then

immersed in visualization solution consisting of 1% DAB in 1xPBS with 0.03% H_2O_2 added just before use and mixed by inverting vigorously. The reaction was stopped after 10 minutes by immersing in 1xPBS. Slides were then given five 5-minute washes in 1xPBS, after which they were dehydrated through an alcohol series (70%, 99% and 95% ethanol, a 50:50 mix of 95% ethanol and xylene and pure xylene) and coverslipped with DPX Mounting Medium.

3.2.6 Designing oligonucleotide probes

The process of designing oligonucleotide probes against mesotocin and vasotocin mRNA is described in Chapter 2.

Briefly, the Primer3 web tool (link given in Chapter 2) was used to design 35-nucleotide-long probes from the chicken vasotocin and mesotocin protein-coding nucleotide sequences. BLASTn was used to confirm that the region used to design the probes matched only the desired peptide. The sequences of the probes used were as follows:

For mesotocin: TTTAATAACATCAAACAACATGGCATTATCTCGGT

For vasotocin: ATTAGATCTGCTATTTACAACGGGGCCTGCTGATG

3.2.7 *In situ* hybridisation (ISH)

The detailed protocol for this procedure, along with a short introduction to the procedure can be found in Chapter 2. What follows is a brief summary.

Pre-selected slides were fixed in 4% paraformaldehyde for 10 minutes. After being washed in 0.01M PBS, the slides were incubated for 10 minutes in Triethanolamine/acetic anhydride (TEA/AA) solution. The slides were rinsed briefly in ddH_2O and then processed through 70% ethanol, 95% ethanol, 100% ethanol, 100% chloroform, and 100% and 95% again for 3 minutes each. Slides were left to dry at RT for 30 min and then incubated overnight at 37°C in oligo probe hybridisation buffer (24% 5M NaCl, 2 % Tris pH 7.6, 2% Denhardt's solution, 0.4 % 250mM EDTA, 10% of 25% Dextran sulphate, 1% of 5% NaPPI, 0.4 % Yeast tRNA, 0.5% Yeast total RNA, 2% salmon testes DNA, 0.66% Poly (A), 50% formamide and topped up to 100% with autoclaved ddH_2O) containing mesotocin or vasotocin sense or antisense probe. After hybridisation was complete, each slide was given three brief rinses in 0.01M SSC at RT, four 15-minute washes in 0.01M SSC at 52°C for mesotocin or 56°C for vasotocin and two thirty-minute washes in 0.01M SSC at RT. Slides were rinsed briefly in ddH_2O and allowed to air dry overnight, after which they were dipped in Ilford K5 Gel Emulsion under safe light conditions and exposed for 18 days at 4˚C. The slides were then developed. Fixative and Phenysol liquid developer (Ilford) were diluted 1 in 5 in ddH_2O before use under safe light conditions. Slides were put in slide racks and immersed once in developer, twice in fixative and three times in ddH_2O for 5 min each before being counterstained in autostainer with hematoxylin "Z", dehydrated through an alcohol series and coverslipped.

No hybridisation signal was detected in slides incubated with the sense/control mesotocin and vasotocin probes (Figure 3.1).

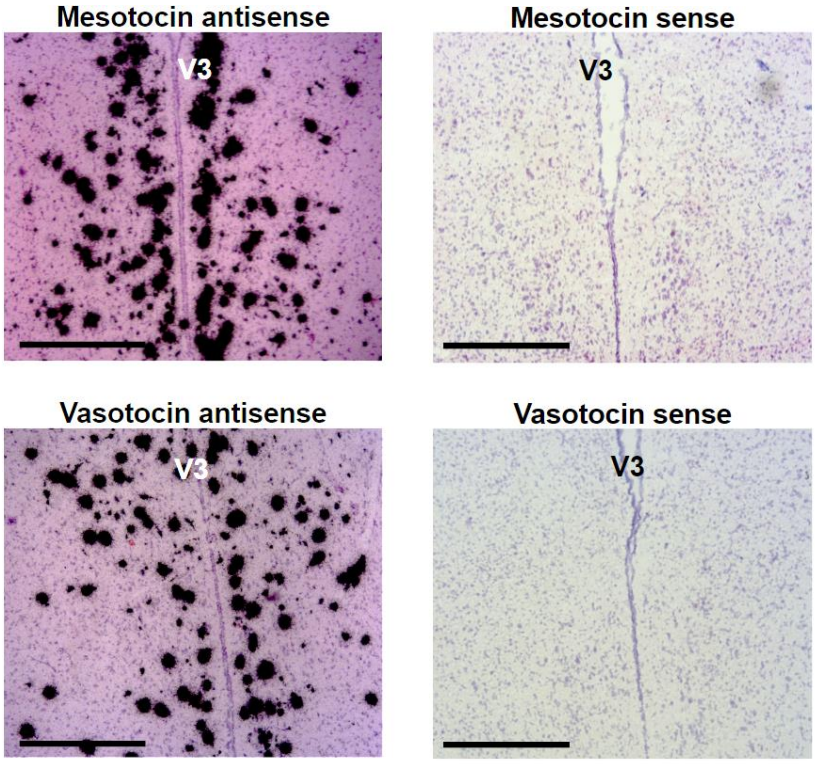

Figure 3.1 Negative control for mesotocin and vasotocin oligoprobes.
Chicken brain sections in the region of the PVN hybridised with antisense (left) and sense (negative control, right) radioactively labelled oligoprobes for both mesotocin (top) and vasotocin (bottom). Positive signal (siver grains, seen as black dots) can be seen on the images on the left hybridised with the antisense probes. No signal was present on sections hybridised with the sense probes. The position of the third ventricle (V3) is shown. Scale bars = 500µm.

3.2.8 Image capture and analysis

Details of the protocol used for image capture and analysis can be found in Chapter 2.

For this experiment all images were captured at a 4x magnification, with all settings kept constant throughout the capturing process. Measurements were taken from 4 hemi-sections for each bird for the BnSTl and 6 hemi-sections for each bird for the PVN. Each two consecutive sections analysed were 45-60µm apart. The density of the mesotocin and vasotocin hybridisation signal was measured in these areas as the % area covered by signal in the region of interest. An average of measurements on the left- and right-hand side of the brain was taken.

3.2.9 Receptor binding autoradiography and binding signal density analysis

The radioactive ligand used in this procedure has been previously validated by the Meddle lab with the addition of unlabelled oxytocin (negative control) to the incubation buffer in order to determine background/non-specific binding. No signal was present on negative control sections (unpublished data).

Pre-selected slides were fixed in 0.1% paraformaldehyde for 5 min. After being washed in Tris buffer, the slides were incubated for 1 hour at RT in the radioactive tracer (^{125}I Ornithine Vasotocin Analog). Incubation was followed by extensive washes in 50mM Tris, 10mM $MgCl_2$ Buffer after which slides were apposed to autoradiographic film. The position of each slide in the

autoradiographic cassette was recorded. Slides were coded so that the experimenter analysing them was unaware of the identity of the birds.

The autoradiographic film was photographed under consistent conditions and the signal density in the LS was analysed on the left- and right-hand side of the brain for each of 3 sections with the MCIDCore software (MCID Software). As only the comparison between groups was of interest, and not any exact values, the software was not calibrated to calculate the actual amount of radioactivity per tissue quantity. Therefore, signal intensity is reported in arbitrary units. A background measurement was also taken from an adjacent arean on each image and subtracted from the LS measurement to ensure that possible differences in background did not interfere with results.

3.2.10 Statistical analysis

Statistical software used was Sigmaplot (Sigmaplot Software Inc). One-way ANOVA and Tukey's post-hoc test (when ANOVA indicated a difference between groups) or Kruskal-Wallis ANOVA on ranks and Dunn's post-hoc test when data did not satisfy the assumptions for ANOVA were performed using treatment as the independent variable. $P<0.05$ was considered statistically significant. An average of all measurements from the left- and right-hand side of the brain for each bird was used in the analysis.

3.3 Results

3.3.1 Body weight and oviduct weight

One-way ANOVA showed that average body weight did not differ significantly between groups ($F_{(3,33)}$=0.954, p=0.426). There was a significant difference in oviduct weights ($F_{(3,33)}$=14.842, p<0.01) at the end of treatment. The oviduct weights of all three steroid-treated groups were significantly different from control (Tukey's test, p<0.001 for all three comparisons) but did not differ significantly between each other (Tukey's test, p>0.05). Results are shown on Figure 3.2 (A and B).

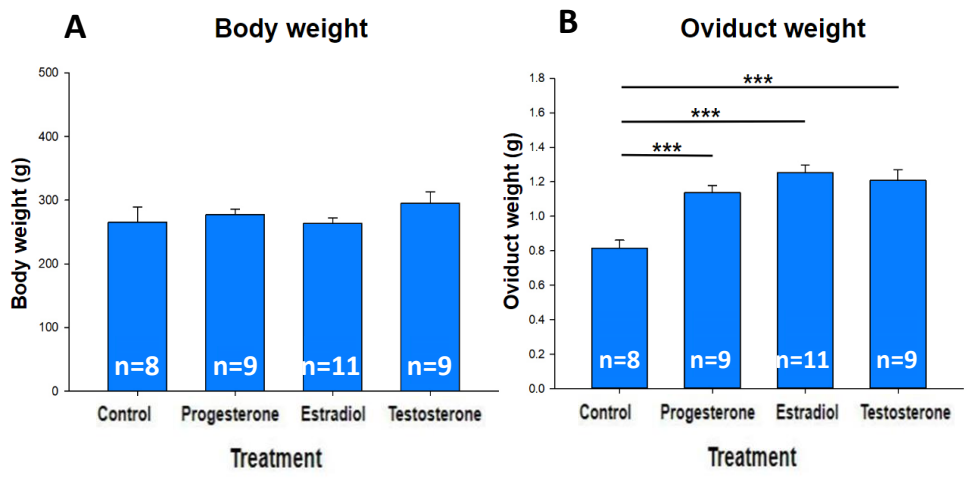

Figure 3.2 Effect of acute injection with sex steroids after a period of priming with DES on the body weight and oviduct weight of juvenile hens.
A. There was no difference in body weight between chicks injected with testosterone, estradiol, progesterone or control (One-way ANOVA, $F_{(3,33)}$=954, p=0.426).
B. Steroid treatment produced a significant increase in the weight of the oviduct compared to control (One-way ANOVA, $F_{(3,33)}$=14.842, p<0.01; Tukey's test, p<0.001 for all three steroids compared to control). n=8-11/group. Some of the birds included in this calculation had to be excluded from later procedures due to their brains being accidentally destroyed during dissection or cryosectioning or the tissue being used to test procedures. *** denotes p<0.001. Data are presented as mean + SEM.

3.3.2 Distribution of mesotocin and vasotocin mRNA-expressing and mesotocin-immunoreactive neurons

Figure 3.3 (A and B) shows the distribution of mesotocin and vasotocin mRNA-expressing neurons observed in this study. At the level of the anterior commissure (CA) and caudal to it, mesotocin and vasotocin mRNA-expressing neurones were present along the third ventricle in the PVN and POM, as well as in the lateral hypothalamus (LHy), and around the lateral and medial forebrain bundle (FPL and FPM). Mesotocin and vasotocin

mRNA-expressing neurones could also be seen in both the medial and lateral BnST. Silver grains were also present in the hyperstriatum ventrale (HV). In sections caudal to the CA mesotocin-immunoreactive cells were observed in the PVN and LHy.

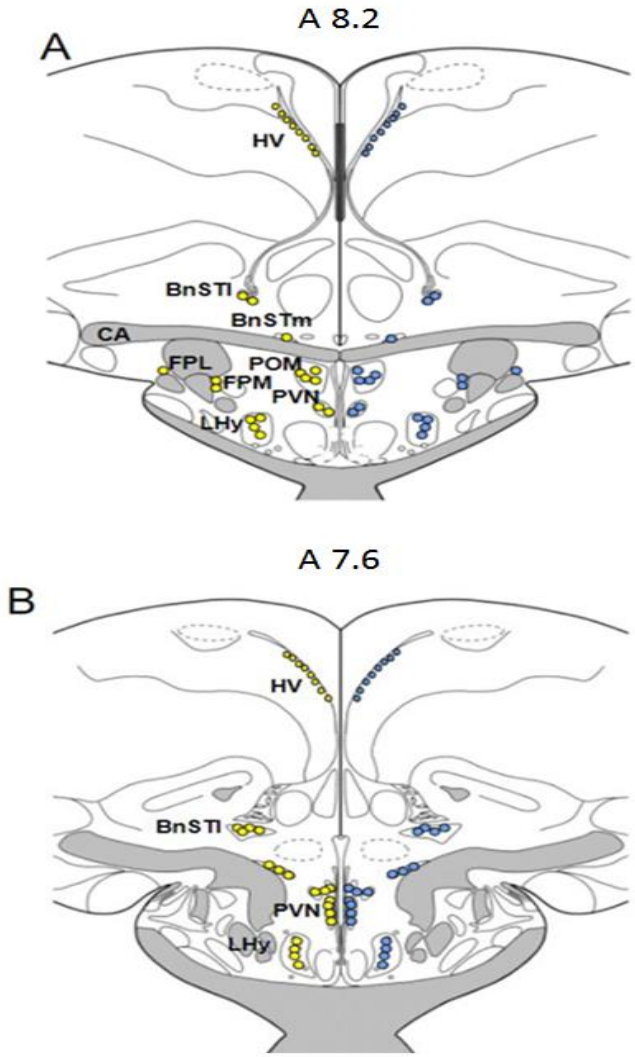

Figure 3.3 Distribution of mesotocin and vasotocin mRNA expression in the hypothalamus of the juvenile hen.
Schematic diagrams of coronal sections illustrating the distribution of mesotocin (yellow dots) and vasotocin (blue dots) mRNA-expressing neurons in the brain of the juvenile hen. The two peptides were present in the same brain areas. Illustrations are adapted, with the given coordinates (A and B, top of each image), from the Stereotaxic Atlas of the Brain of the Chick (Kuenzel and Masson, 1988), where 'A' followed by a number signifies a brain plate the given number of millimetres anterior to a zero reference plane. LHy, Regio lateralis hypothalami (lateral hypothalamic area); PVN, nucleus paraventricularis magnocellularis; V3, ventriculus tertius (third ventricle), anterior commissure (CA), POM (medial preoptic area), lateral and medial forebrain bundle (FPL and FPM), medial bed nucleus of the stria terminalis (BnSTm), lateral bed nucleus of the stria terminalis (BnSTl), hyperstriatum ventrale (HV).

3.3.3 Effects of steroid administration on mesotocin and vasotocin mRNA expression in the PVN and BnSTl

Figures 3.4 (A and B) and 3.5 (A, B and C) respectively show photomicrographs together with graphical representations of the effects of treatment on mesotocin mRNA expression in the PVN and BnSTl after each of the treatments. Kruskal-Wallis ANOVA on ranks revealed significant differences in mesotocin mRNA expression between groups in both the PVN ($H_{(3)}$=8.992, p=0.029) and BnSTl ($H_{(3)}$=11.065, p=0.011). Dunn's test confirmed that the testosterone-treated group significantly differred from control and had higher mRNA expression in the PVN (p=0.033) (Figure 3.4 B). In the BnSTl, the testosterone-treated group was significantly different from the estradiol-treated group explaining the ANOVA result (Dunn's test, p=0.008, difference not marked on figure as not relevant to the question of the experiment) but none of the groups were significantly different from control (Figure 3.5 B). However, when only the testosterone-treated and control group were analysed together with Student's t-test (Figure 3.5 C), a significant difference was found with the testosterone group showing higher mRNA expression than control (two-tailed, $t_{(8)=}$ -2.807, p=0.023).

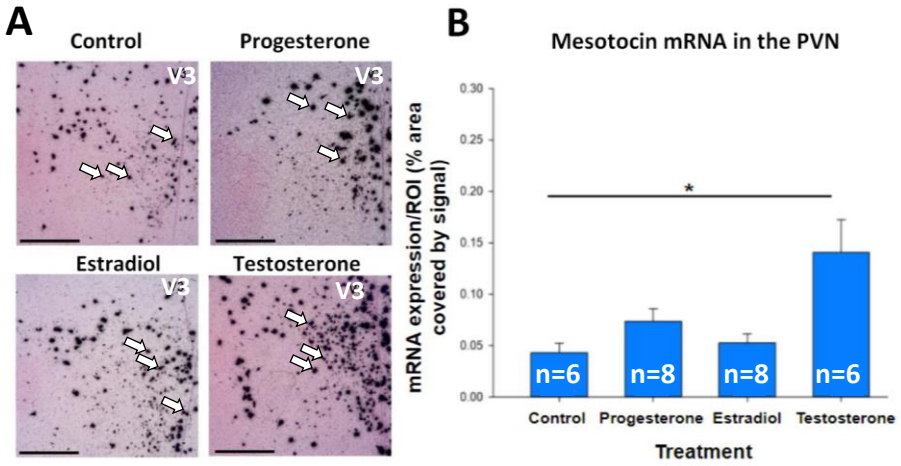

Figure 3.4 Mesotocin expression in the PVN of juvenile hens injected with sex steroids after a period of priming with DES.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the left-hand side PVN of chicks injected with corn oil control, progesterone, estradiol or testosterone. Arrows denote examples of labelled cells. The third ventricle (V3) is shown. Scale bar = 500µm.
B. There was a significant difference in the mesotocin mRNA expression measured as the % area covered by signal in the region of interest (ROI) in the PVN between chicks injected with testosterone, estradiol, progesterone or corn oil control (Kruskal-Wallis ANOVA on ranks, $H_{(3)}$=8.992, p=0.029). Testosterone-treated birds had significantly higher mRNA expression than control (Dunn's test, p=0.033). n=6-8 (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal). An average of measurements from the left- and right-hand side of the brain was taken for each bird. * denotes p<0.05. Data are presented as mean + SEM.

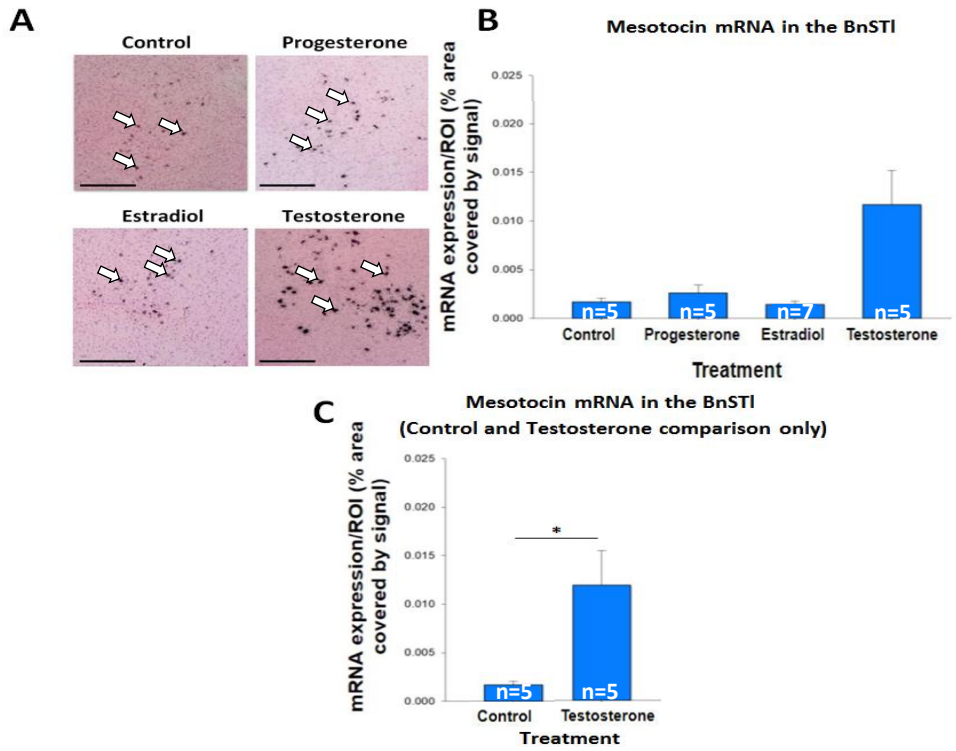

Figure 3.5 Mesotocin expression in the BnSTl of juvenile hens injected with sex steroids after a period of priming with DES.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the left-hand side BnSTl of chicks injected with corn oil control, progesterone, estradiol or testosterone. Arrows denote examples of labelled cells. The left-hand side ventricle is not shown on the images but is positioned directly above. Scale bar = 250µm.
B. There was a significant difference detected between groups in the mesotocin mRNA expression measured as the % area covered by signal in the region of interest (ROI) in the BnSTl for chicks injected with testosterone, estradiol, progesterone or corn oil control (Kruskal-Wallis ANOVA on ranks, $H_{(3)}$=11.065, p=0.011), however, this difference was between the testosterone-treated and estradiol-treated groups (Dunn's test, p=0.008) and is therefore not marked on the image above (B) as it is not relevant to the experimental question.
C. When the testosterone-treated and control group were compared with Student's t-test a significant difference was found with the testosterone-treated group showing higher mRNA expression ($t_{(8)}$=-2.807, p=0.023). n=5-7/group. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal. In two cases, I had not chosen the slides accurately enough so the part of the region visible on sections from those birds did not match the rest of the birds and they were also excluded from the analysis.) An average of measurements on the left- and right-hand side of the brain was taken for each bird. * denotes p<0.05. Data are presented as mean + SEM.

Figures 3.6 (A and B) and 3.7 (A and B) respectively show photomicrographs together with graphical representations of the effects of treatment on vasotocin mRNA expression in the PVN and BnSTl after each of the treatments.

No difference between groups was found in the PVN with One-way ANOVA ($F_{(3,24)}$=0.281, p=0.839). Kruskal-Wallis ANOVA on ranks found a significant difference in the BnSTl ($H_{(3)}$=15.776, p=0.001). Both testosterone and estradiol increased vasotocin mRNA mRNA expression in the BnSTl compared to control (Dunn's test, p=0.015 for testosterone vs control and p=0.047 for estradiol vs control) and the testosterone-treated and estradiol-treated groups were not significantly different from each other (Dunn's test, p>0.05).

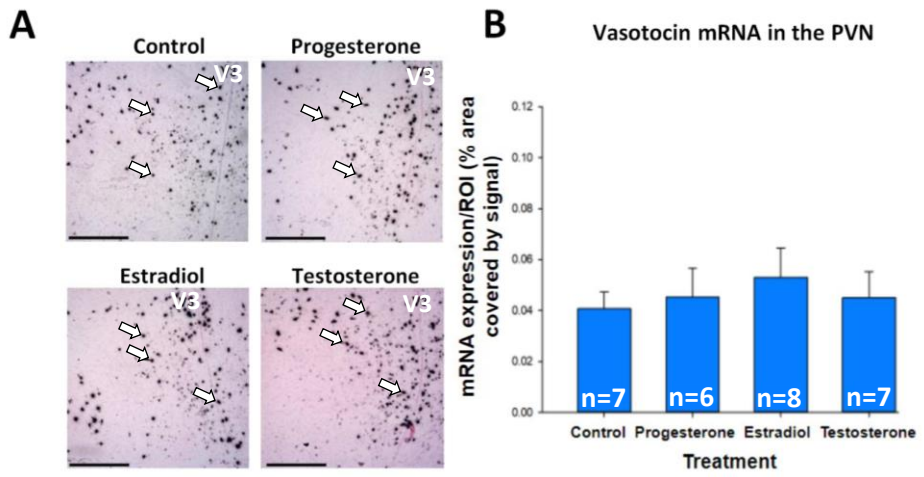

Figure 3.6 Vasotocin expression in the PVN of juvenile hens injected with sex steroids after a period of priming with DES.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the left-hand side PVN of chicks injected with corn oil control, progesterone, estradiol or testosterone. Arrows denote examples of labelled cells. The third ventricle (V3) is shown. Scale bar = 500µm.
B. There was no significant difference in the vasotocin mRNA expression measured as the % area covered by signal in the region of interest (ROI) between chicks injected with testosterone, estradiol, progesterone or corn oil control (One-way ANOVA, $F_{(3,24)}$=0.125, p=0.945). n=6-8/group. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal.) An average of measurements on the left- and right-hand side of the brain was taken for each bird. Data are presented as mean + SEM.

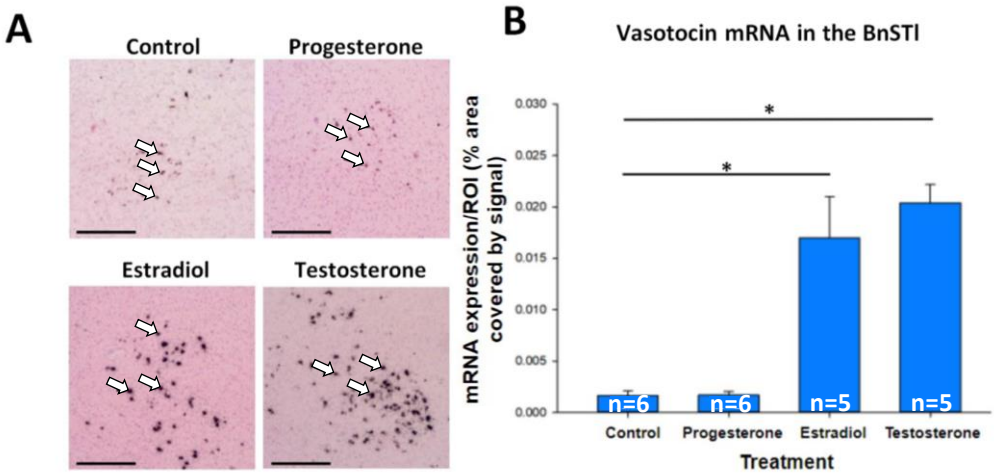

Figure 3.7 Vasotocin expression in the PVN of juvenile hens injected with sex steroids after a period of priming with DES.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the left-hand side PVN of chicks injected with corn oil control, progesterone, estradiol or testosterone. Arrows denote examples of labelled cells. The third ventricle (V3) is shown. Scale bar = 500µm.
B. There was a significant difference in the vasotocin mRNA expression in BnSTl measured as the % area covered by signal in the region of interest (ROI) between chicks injected with testosterone, estradiol, progesterone or corn oil control (Kruskal-Wallis ANOVA on ranks, $H_{(3)}$=15.776, p=0.001). Both testosterone and estradiol increased vasotocin mRNA mRNA expression in the BnSTl compared to control (Dunn's test, p=0.015 for testosterone vs control and p=0.047 for estradiol vs control). n=6-8/group. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal.) An average of measurements on the left- and right-hand side of the brain was taken for each bird. Data are presented as mean + SEM.

3.3.4 Effects of steroid administration on mesotocin immunoreactivity in the PVN

Figure 3.8 shows a schematic representation of the distribution of mesotocin-immunoreactive neurones in the examined part of the brain.

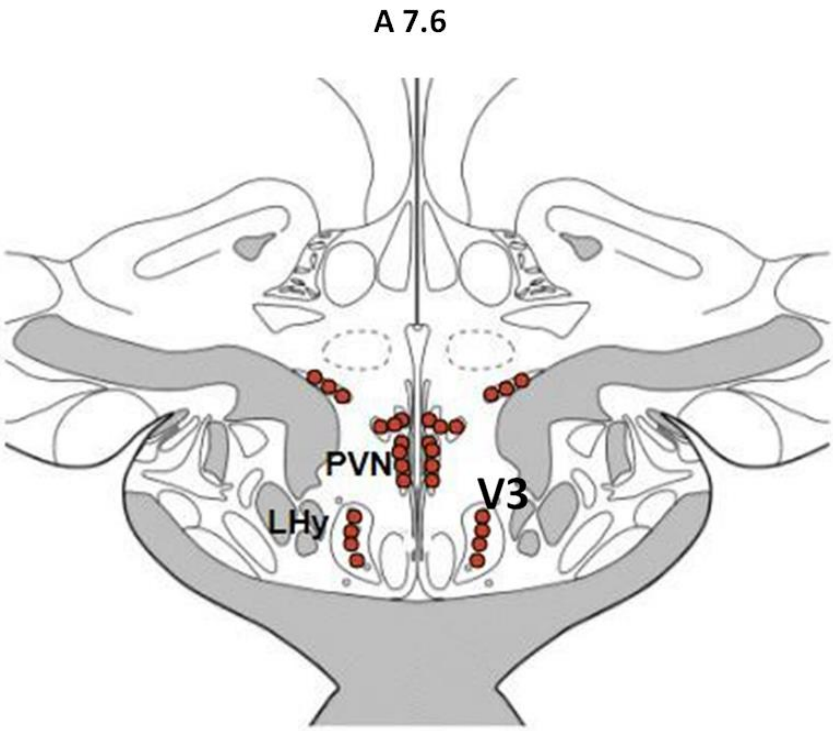

Figure 3.8 Distribution of mesotocin immunoreactivity in the hypothalamus of the juvenile hen.
Schematic diagram of coronal sections illustrating the distribution of mesotocin immunoreactivity (red dots) in the brain of juvenile hens. Illustration is adapted, with the given coordinates (top of image), from Stereotaxic Atlas of the Brain of the Chick (Kuenzel and Masson, 1988), where 'A' followed by a number signifies a brain plate the given number of millimetres anterior to a zero reference plane. LHy, Regio lateralis hypothalami (lateral hypothalamic area); PVN, nucleus paraventricularis magnocellularis; V3, ventriculus tertius (third ventricle).

Figure 3.9 (A, B and C) shows photomicrographs along with a graphical representation of the effect of treatment on mesotocin immunoreactivity in the PVN of juvenile hens after each of the treatments.

Mesotocin immunoreactivity was measured similarly to mRNA expression as the % area covered by signal in the medial part of the PVN. An average of measurements on the left- and right-hand side of the brain was taken. One-

way ANOVA showed no difference between treatments ($F_{(3,21)}$=2.088, p=0.132). However, Student's t-test between the control and testosterone-treated groups showed a significant difference ($t_{(10)=}$3.924, p=0.003) with the testosterone group having lower levels of immunoreactivity than control.

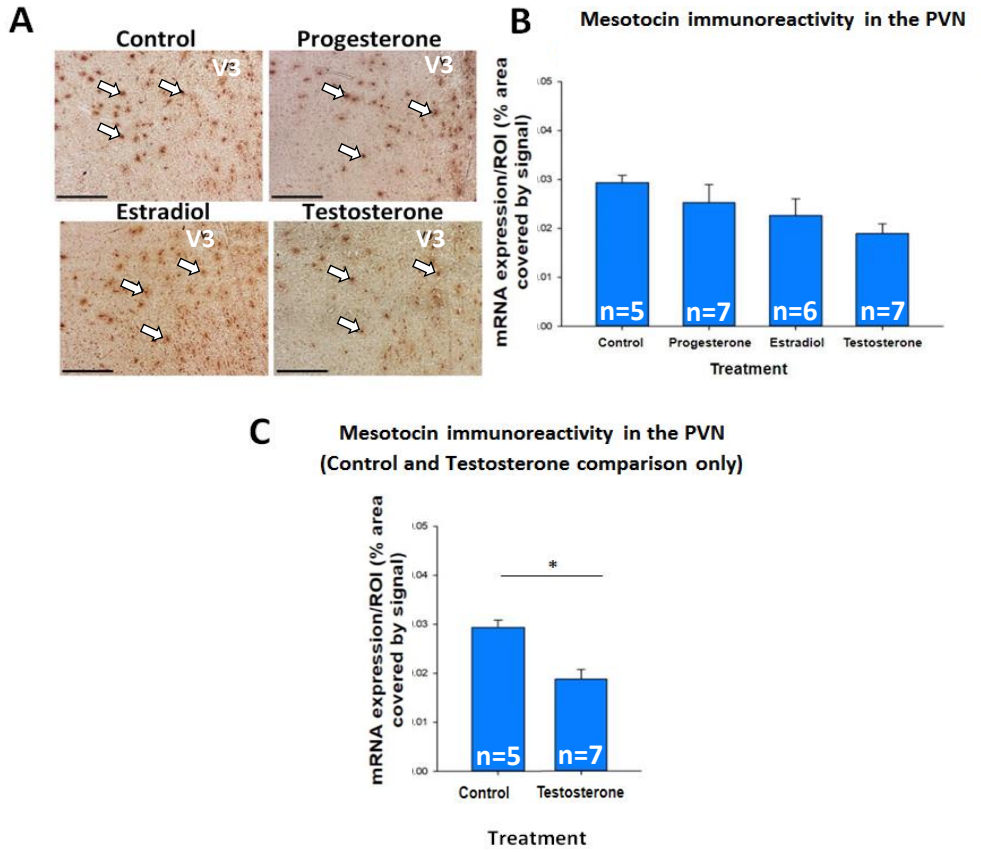

Figure 3.9 Mesotocin immunoreactivity in the PVN of juvenile hens injected with sex steroids after a period of priming with DES.
A. Photomicrographs show immunostained neurons in the left-hand side PVN of chicks injected with corn oil control, progesterone, estradiol or testoterone. Arrows denote examples of labelled cells. The third ventricle (V3) is shown. Scale bar = 250µm.
B. One-way ANOVA showed no significant difference in mesotocin immunoreactivity measured as the % area covered by signal in the region of interest (ROI) in the PVN between chicks injected with corn oil control, progesterone, estradiol or testosterone when all groups were tested together (One-way ANOVA, $F_{(3,21)}$=2.088, p=0.132).
C. When the testosterone-treated and control group were compared with Studen't t-test, a difference was found ($t_{(10)}$=3.924, p=0.003) with the testosterone-treated group showing lower levels of immunoreactivity than control.
n=5-7/group. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to damage to sections. As the sectioned used in this procedure were thinner than the optimal thickness for immunohistochemistry, the tissue was damaged easily.)
An average of measurements on the left- and right-hand side of the brain was taken for each bird. Data are presented as mean +SEM.

3.3.5 Distribution of mesotocin receptor binding in the juvenile chicken and effects of steroid administration on mesotocin receptor binding in the lateral septum

Figure 3.10 shows photographs of mesotocin receptor binding throughout the brain of a male juvenile domestic chick.

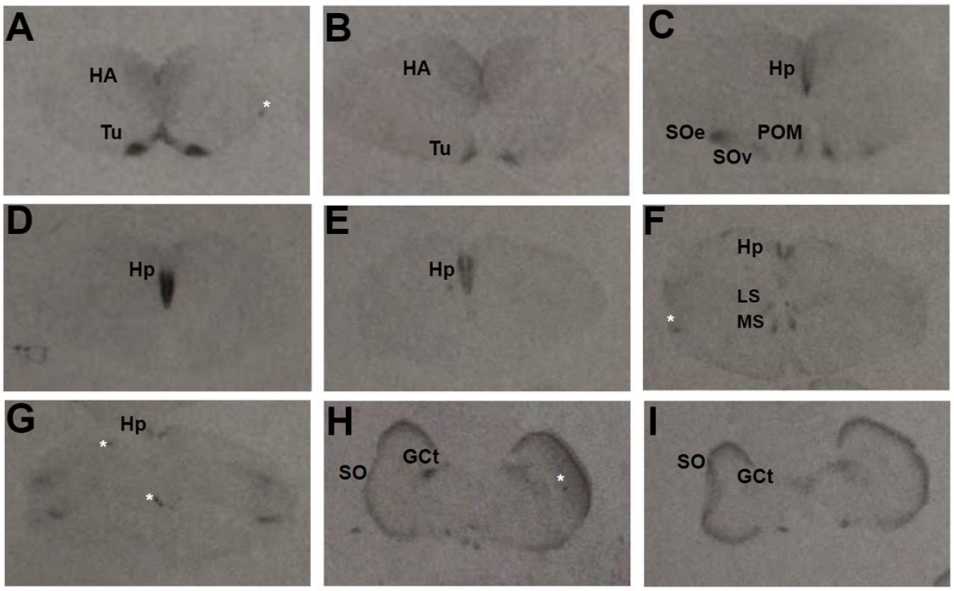

Figure 3.10 Photographs showing mesotocin radioactive ligand receptor binding signal throughout the brain of a juvenile male chick.
Identifiable areas where signal is present are labelled of the left-hand side of sections. Signal was present in the hyperstriatum accessorium (HA), tuberculum olfactorium, hippocampus (Hp), POM, supraoptic nucleus (SOe and SOv), lateral and medial septum (LS and MS), periaqueductal gray (GCt) and optic striatum (SO). Other areas which could not be clearly identified. White asterisks denote areas where dark spots are seen due to a dirt particle or defect on the slide rather than real signal. Areas were identified with the help of the stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988).

Figure 3.11 (A-D) shows a schematic representation of the distribution of binding signal for the mesotocin receptor.

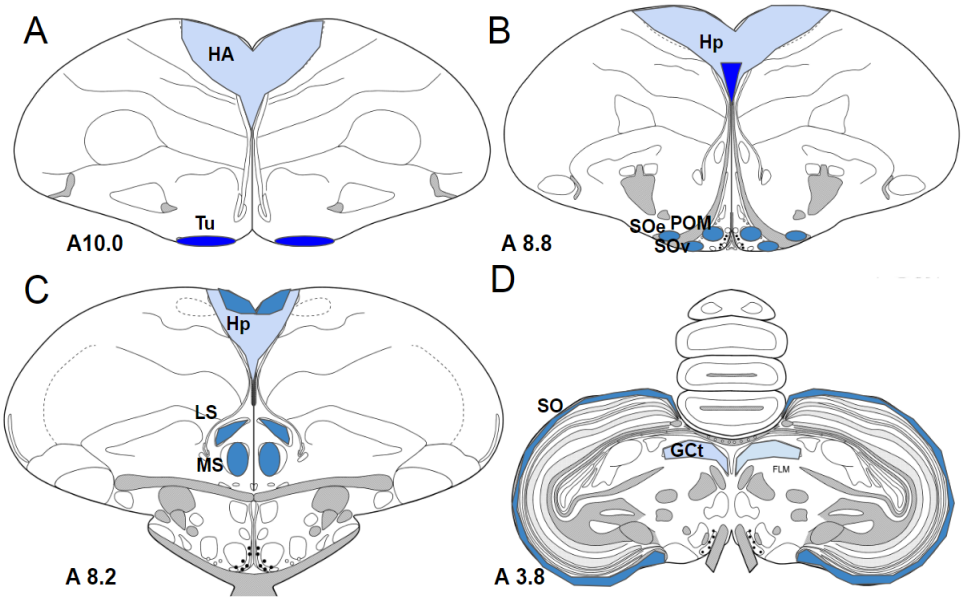

Figure 3.11 Schematic diagrams of coronal section representing the distribution of mesotocin receptor binding (areas coloured in shades of blue) in the brain of the domestic chick based on the autoradiographic study shown on Figure 3.10.
The intensity of the blue colour represents the intensity of signal. Presence of binding is shown in the hyperstriatum accessorium (HA), tuberculum olfactorium (Tu), hippocampus (Hp), medial preoptic area (POM), supraoptic nucleus (SOe and SOv), lateral and medial septum (LS and MS), periaqueductal gray (GCt) and optic striatum (SO). Illustrations are redrawn, with the given coordinates (lower left corner of each image), from Stereotaxic Atlas of the Brain of the Chick (Kuenzel and Masson, 1988), where 'A' followed by a number signifies a brain plate the given number of millimetres anterior to a zero reference plane.

Autoradiographic signal was observed in the hyperstriatum accessorium (HA), tuberculum olfactorium (Tu), hippocampus (Hp), POM, nucleus supraopticus, pars externus and nucleus supraopticus pars ventralis (SOe and SOv), lateral and medial septum (LS and MS), periaqueductal gray (GCt), optic striatum (SO) and in several other areas which could not be clearly identified.

Binding signal in the LS was visible on slides just prior to the appearance of the anterior commissure (CA) but once the CA appeared signal became extremely faint or non-existent. These observations were consistent across all four groups. This suggests that mesotocin (VT3, OTR-like) receptors are only present in the rostral part of the LS in the juvenile chicken. With this in mind, only sections just prior to the CA were included in later analysis. Figure 3.12 shows photographs of mesotocin receptor binding in the LS of birds after each of the treatments, along with a graphical representation of the effects of treatment on receptor binding in this area. One-way ANOVA revealed no significant effect of treatment on the density of mesotocin binding signal in this part of the lateral septum ($F_{(3,19)}$=2.118, p=0.132).

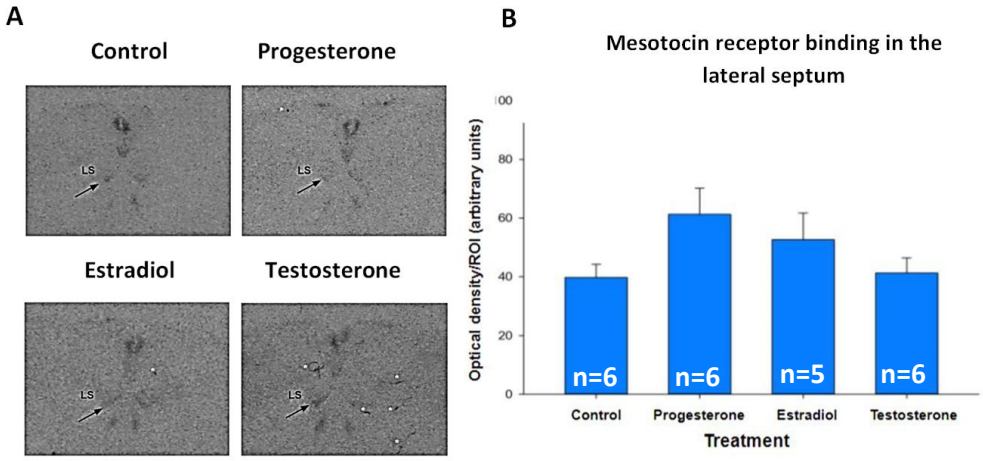

Figure 3.12 Mesotocin radioactive ligand receptor binding signal in the lateral septum (LS) of juvenile hens injected with sex steroids after a period of priming with DES.
A. Photographs showing radioactive ligand receptor binding signal in the lateral septum. Arrows and labels denote the LS on the left-hand side of each section. White asterisks denote areas where dark spots are seen due to a dirt particle or defect on the slide rather than real signal. The position of the sections in the brain and the distribution of the signal corresponds to that shown in Figure 3.10 F and 3.11 C.
B. There was no significant difference in the mesotocin receptor binding measured as the optical density of signal in arbitrary units in the LS between chicks injected with testosterone, estradiol, progesterone or corn oil control (One-way ANOVA, $F_{(3\text{-}19)}$=2.118, p=0.132). n=5-6/group (8 birds per group were included in the procedure but some individuals were taken out of the analysis due to tissue damage obscuring signal or due to sections being lost during cryosectioning. As signal was only visible in a small part of the LS, the accidental loss of a small number of sections during cryosectioning sometimes meant that a bird could not be analysed.) An average of measurements on the left- and right-hand side of the brain was taken for each bird. Data are presented as mean + SEM.

3.4 Discussion

3.4.1 Distribution of mesotocin and vasotocin mRNA-expressing cells and mesotocin-immunoreactive cells

The distribution of mesotocin and vasotocin mRNA-expressing and mesotocin-immunoreactive cells observed in this study did not show any marked differences to the general mesotocin/vasotocin neuronal distribution reported in previous studies in the chicken or other birds (Aste et al., 1996; Aste et al., 1998; Barth et al., 1997; Chaturvedi et al., 1994; Robinzon et al., 1988) with the majority of mRNA expression seen in areas of the neural behaviour network. Some mesotocin and vasotocin signal was observed along the upper ventricles in the hyperstriatum ventrale (HV). This is a region which has been shown to be involved in the process of imprinting in chicks (Horn et al., 1979). Imprinting involves memorising the characteristics of the first moving object a chick sees directly after hatch and leads to the chick then displaying preference for that object (Nakamori et al., 2013). As nonapeptides are known to play roles in learning and memory (Bohus et al., 1978; Davis and Pico, 1984), it is possible that the presence of mesotocin and vasotocin expressing neurons in this brain area may be related to this learning process. To the author's knowledge, these peptides have not been studied before in relation to imprinting so further investigation is needed to determine whether there is a connection. Measuring nonapeptide expression in the HV of chicks which have been induced to imprint or prevented from imprinting on an object could provide some useful insights.

As expected, mesotocin-immunoreactive neurons caudal to the CA were seen mainly in the PVN and lateral hypothalamus (LHy) and matched the pattern of mesotocin mRNA-expressing cells in these areas. These neurons were visible and quantifiable in the PVN which was the area of interest for

this study. However, as the 15µm thick sections used in order to be able to perform both ISH and IHC were less than the optimal thickness for this procedure it was difficult to determine the position of any mesotocin fibres and it is possible that less strongly stained neurons would not have been detected. Therefore, the distribution described here should not be considered an exhaustive representation of all mesotocin-immunoreactive neurons in this part of the chicken brain.

3.4.2 Effect of gonadal steroids on vasotocin mRNA expression

The results of this study, which show that both testosterone and estradiol increased vasotocin mRNA in the BnSTl, are similar to those seen in quail for the medial part of the BnST (Aste et al., 2013; Balthazart et al., 1998; Voorhuis and de Kloet, 1992). To the author's knowledge, the effect of sex steroids on the BnSTl vasotocin expression is demonstrated for the first time in birds. The BnSTl has been implicated in stress in both birds and mammals and vasotocin has been shown to be involved in the regulation of corticosterone, the main avian stress hormone (Cecchi et al., 2002; Nagarajan et al., 2014; Selvam et al., 2013), therefore it is possible that the vasotocin neurons in this brain area are involved in the stress response. These results also support previous work which has shown interactions betweensex sterodis and the stress network (Critchlow et al., 1963; Dickens and Bentley, 2014; Handa et al., 2011; Klukowski et al., 1997; Larkin et al., 2010; Nagra et al., 1965; Seale et al., 2005a; Seale et al., 2005b; Zysling et al., 2006). It has been demonstrated in rats that high levels of estrogen may be correlated with higher sensitivity to stress (Crofton et al., 1985), which would be consistent with the increased vasotocin expression seen with estradiol treatment in this study. Interestingly, treatment with testosterone also increased vasotocin in this brain area and testosterone has been shown

to lower stress sensitivity through lowering the corticosterone stress response in rodents (Viau and Meaney, 2004). One explanation for the results observed in the present study is that it is not testosterone itself but its metabolite estradiol that caused the observed effect. The enzyme aromatase converts testosterone to estradiol and aromatase-immunoreactive cells have been co-localised in the avian brain with vasotocinergic neurons (Balthazart et al., 1997a). Studies in quail support the hypothesis that testosterone acts through its metabolite after aromatisation (Beyer et al., 1994; Foidart et al., 1994; Panzica et al., 2001; Viglietti-Panzica et al., 2001). The data from this study are consistent with those findings (as both hormones affected vasotocin mRNA expression to similar extents) and suggest that in the female chicken BnSTl vasotocin mRNA expression may be affected by ovarian estradiol or by estradiol produced in the brain after aromatisation of testosterone. Administering testosterone with and without aromatase inhibitors and observing the effects on vasotocin cells in the BnSTl could help confirm this hypothesis. The mammalian vasotocin gene is known to contain estradiol response elements which may be involved in regulating the effects of estradiol on vasotocin transcription, but it is not known whether similar elements exist in the bird vasotocin gene (Shapiro et al., 2000). In mammals, estrogen receptors are present in the PVN and are co-localised with nonapeptide neurons (Somponpun and Sladek, 2003) but no androgen receptors have been reported in that area. ER-β but not androgen receptors have been found the BnSTm of quail and while neither estrogen nor androgen receptors were mapped specifically to the PVN in quail, both ER-α and ER-β were found in a non-specified area located between the POM and the caudal part of the tuberal hypothalamus (Voigt et al., 2009). Further investigation is needed to determine the distribution of these receptors in the chicken and their potential co-localisation with nonapeptide neurons. Similarly, while it is possible that the higher vasotocin mRNA expression in the BnSTl after both testosterone and estradiol treatment may be connected to the stress response, further studies are needed to test this, especially as

stress was not measured in the experiments presented here. An ISH study examining the expression of BnSTl vasotocin neurons under different stress conditions could help confirm this hypothesis. In addition, as the stress response has been shown to change with puberty, experiments with both juvenile and adult birds could provide a more detailed picture of the role of this nucleus. Furthermore, the involvement of testosterone and estradiol needs to be more closely studied with experiments examining the behavioural stress response together with the effects of sex steroids on the vasotocin innervation in this nucleus.

Previous findings in the quail PVN show that administration of estradiol or testosterone upregulated vasotocin immunoreactivity in that brain region (Aste et al., 2013). Therefore, it was somewhat surprising that in this study, none of the steroids had a significant effect on the vasotocin mRNA expression in the PVN. However, as the upregulation in the quail was observed after steroid injection on 16 consecutive days, it is likely that a single acute injection, albeit after a period of priming, was not enough to induce the response.

3.4.3 Effect of gonadal steroids on mesotocin mRNA expression

Data on the effects of sex steroids on the mesotocin system in birds are not available, to the author's knowledge. The results of this study show for the first time that acute administration of testosterone is capable of increasing mesotocin mRNA expression in the PVN and BnSTl of treated birds while progesterone and estradiol had no significant effect. This result suggests that testosterone may affect mesotocin expression directly and not through its estradiol metabolite (Beyer et al., 1994; Foidart et al., 1994; Panzica et al., 2001; Viglietti-Panzica et al., 2001). This is surprising, as a study on the distribution of gonadal steroid receptors in the quail found no androgen

receptors in the PVN or BnSTm (Voigt et al., 2009). This suggests that the effect may be mediated by cells in other parts of the brain which have connections to PVN and BnSTl mesotocinergic neurons. It is also possible that there is a difference in receptor distributions between the chicken and quail and androgen receptors are present in these brain areas in the chicken, even though the two species are closely related. Therefore, the mechanism through which testosterone may affect mesotocin expression in these brain areas may be further studied through an investigation of the distribution of androgen receptors in the chicken and the afferent connections to the areas of interest, as well as through administering sex steroid receptor antagonists and observing whether they alter the effects of testosterone on mesotocin mRNA expression.

3.4.4 Effect of gonadal steroids on mesotocin immunoreactivity in the paraventricular nucleus

When all groups were analysed together, no significant effect of treatment on mesotocin-immunoreactive neurones in the PVN was found, despite the increase in mesotocin mRNA in that brain area with the administration of testosterone. However, comparing the testosterone-treated and control groups with Student's t-test showed that immunoreactivity was lower in the testosterone-treated group. This was surprising, considering the observed increase in mRNA expression in the same area after testosterone injection. It is possible that less immunoreactivity in the testosterone group may be caused by mesotocin being quickly transported out of cells. This sort of discrepancy has been observed before in a study in rats where estradiol treatment in the MPOA increased oxytocin mRNA expression but oxytocin perikarya in the area decreased (Caldwell et al., 1988). In the present study, brains were collected a short time after the steroid injection. It is possible

that, given more time, the protein would have had a chance to accumulate. Studies on vasotocin in rodents show that vasotocin sometimes took days or weeks to accumulate (Miller et al., 1992), and in quail, chronic administration of testosterone eventually but not immediately recovered immunoreactivity in male BnSTm after castration (Viglietti-Panzica et al., 1994). However, it is not known whether this is true for mesotocin in chickens.

Finally, as mentioned before, the sections used in the IHC procedure were not of optimal thickness leading to the experimenter only being able to quantify strongly stained neurons while more subtle differences between groups may have remained undetected. A separate experiment with a similar design but focussing specifically on immunoreactivity with all conditions optimised for this procedure might make signal stronger and results easier to observe and analyse.

3.4.5 Distribution of receptor binding in the brain of the juvenile chicken

The distribution of mesotocin receptors in the chicken brain observed in this chapter differed significantly from that previously reported in zebra finch and white crowned sparrow (Leung et al., 2009). However, this was not surprising as distribution of this receptor showed significant differences between those two songbird species as well, and oxytocin receptor distribution has been known to vary between mammalian species (Insel and Shapiro, 1992). Somewhat surprising was the low intensity of signal in the LS which is a major site of action for mesotocin, and the fact that this signal was confined to only a few sections rostral to the CA, even though the nucleus spans a larger region. It is possible that this faint staining is due to the young age of the birds used in these experiments. To the author's knowledge, the distribution of mesotocin receptor in the adult chicken has not been reported. Very strong signal was present in the hippocampus (Hp) and olfactory

tubercle (Tu). The Hp has been demonstrated to be involved in spatial cognition in mammals (Morris et al., 1982), birds (Balda and Kamil, 1992), reptiles, and fish (Rodríguez et al., 2002). There is evidence that the hippocampus is larger in birds that cache food than in other species and damage to this area interferes with spatial memory (Biegler et al., 2001; Morris et al., 1982). As oxytocin is known to be involved in memory (Bohus et al., 1978) it is possible that mesotocin in the chick also has similar functions through its receptors in this area. The Tu receives input from the olfactory receptors and is known to be involved in processing sensory and social stimuli in rats (Hitt et al., 1973). Lesions in this region in rats caused a reduction in copulation as well as some stereotyped behaviours such a sniffing and chewing (Koob et al., 1978). The Tu also has connections to the reward centers in the brain and may play a role in mediating the link between sensory inputs and the resultant behavioural responses (Ikemoto, 2003; for review, see Wesson and Wilson, 2011). The data presented in this chapter suggest that the involvement of this region in social responses may be mediated by mesotocin through its receptors.

3.4.6 Effect of gonadal steroids on mesotocin receptor binding in the lateral septum

No difference in mesotocin receptor binding in the LS was found in this study between groups. While it has been demonstrated in previous studies in both mammals and birds that testosterone can increase nonapeptide innervation in this brain area, this was not always coupled with detectable changes in receptor binding (Voorhuis et al., 1988a; Voorhuis et al., 1988b). In future studies, it will be beneficial to examine the effects of gonadal steroids on nonapeptide receptor binding in this brain region in adult chickens, perhaps with chronic as well as acute administration. Unfortunately, the attempts of

the Meddle lab to find an adequate ligand and develop a usable procedure for the autoradiographic detection of vasotocin binding in the chicken brain have been unsuccessful so far but efforts should continue in this direction so that vasotocin binding can also be examined.

Conclusion

This study analysed the effects of acute treatment with testosterone, progesterone and estradiol on the mesotocin and vasotocin mRNA expression in the PVN and BnSTl, as well as the mesotocin immunoreactivity in the PVN and the mesotocin receptor binding in the LS of juvenile hens. It was discovered that both testosterone and estradiol significantly increased the vasotocin mRNA in the BnSTl but had no effect in the PVN. The data presented in this chapter suggest that vasotocinergic neurones within the BnSTl are affected by treatment with sex steroids in a similar way to those in the BnSTm (Plumari et al., 2004; van Leeuwen et al., 1985; Viglietti-Panzica et al., 1994; Voorhuis et al., 1988b). These data are also in agreement with the hypothesis that testosterone affects vasotocin expression in the brain through its estrogenic metabolite estradiol (Aste et al., 2013; de Vries et al., 1986; Grassi et al., 2013; Johnson et al., 1991; Patisaul et al., 2003; Viglietti-Panzica et al., 2001). The present experiments also provided the first (to the author's knowledge) data on the previously unexamined interactions between sex steroids and mesotocin in the avian brain. Testosterone alone significantly increased mesotocin mRNA in both the PVN and BnSTl, suggesting that the effects of testosterone on this peptide are direct and not through estradiol. However, as no androgen receptors have been discovered in the brain areas examined here the mechanism through which this effect is achieved remains unknown. Based on previous studies of the HV (Horn et al., 1979; Nakamori et al., 2013), a possible role for vasotocin and mesotocin

in imprinting was suggested here, as hybridisation signal for both peptides was observed in that area. No difference in mesotocin immunoreactivity in the PVN was found when groups were analysed together by ANOVA, but Student's t-test showed significantly less mesotocin immunoreactivity after testosterone treatment. This did not agree with the increase in mesotocin mRNA in the same area with this treatment, but the discrepancy may be due to the peptide being quickly removed from cells. The distribution of mesotocin receptor binding in the brain of the juvenile chick was mapped to several brain regions involved in social and memory functions (Hitt et al., 1973; Koob et al., 1978; Morris et al., 1982; Balda and Kamil, 1992; Rodríguez et al., 2002; Biegler et al., 2001). Therefore, it was proposed here that mesotocin receptors may be involved in the interface between olfactory cues and behavioural responses in the Tu and spatial memory in the Hp in addition to their social functions in areas of the neural behaviour network. The mesotocin binding in the LS showed no differences between groups suggesting no effect of acute sex steroid administration in this region. Overall, these results complement previous data on the control of brain nonapeptides by gonadal steroids while also providing new information on their effects on a peptide and a brain area which had been neglected in other studies and laying the groundwork for further investigation.

Chapter 4 Mesotocin and Vasotocin mRNA Expression in the Brain of the Domestic Hen Throughout the Reproductive Cycle

Abstract

As established in previous chapters, the neuropeptides oxytocin and vasopressin are important regulators of mammalian maternal behaviour, as well as the stress response, and there is evidence that their avian orthologues mesotocin and vasotocin play similar roles in birds. The possible role of vasotocin in maternal behaviour in avian species has not been studied but vasotocin is a known component of the stress axis in the avian brain. Some bird species display an attenuated stress response during certain periods of their reproductive cycle and this lower reactivity to stress is likely a mechanism to ensure that parental birds will prioritise reproduction and care for the offspring over their individual safety.

Studies in the chicken and turkey have now demonstrated that mesotocin is crucial for the care of chicks after hatch as mesotocin immunoreactivity was significantly greater in the PVN and POM of rearing compared to laying hens and mesotocin antagonists completely abolished rearing behaviour in the turkey.

In this study *in situ* hybridisation (ISH) was used to observe the changes in mesotocin and vasotocin mRNA expression in the medial PVN, the SON, the POM, the BnSTm and the BnSTl throughout the reproductive cycle of the domestic hen. Brains were collected from laying hens (Laying), hens at the onset of incubation, taken as the third day of continuously sitting on eggs (Onset), birds on the fourteenth day of incubation (Incubating) and birds

which had hatched chicks and had been rearing them for one day (Rearing). In the medial PVN, rearing birds showed higher mesotocin mRNA expression than hens laying eggs. In one subdivision of the lateral BnST where it was possible to quantify expression, laying and rearing hens showed similar levels of mesotocin mRNA expression while levels for Onset and Incubating were also smilar but significantly lower than for both Laying and Rearing, suggesting that mesotocin mRNA expression in this area decreased during incubation and increased again at the onset of rearing. In another population of cells in the same nucleus, mesotocin expression could only be observed in laying hens, once again suggesting that expression of this peptide decreased with the onset of incubation. This decrease may be related to a decrease in social interaction in incubating hens, as they tend to primarily stay in the nest during incubation and discourage others from approaching, which could mean fewer interactions with conspecifics. Unfortunately, social interaction was not measured in this study. There was no difference between groups in the vasotocin mRNA expression in the PVN, but in the BnSTl (both neuronal populations observed) birds in the Onset, Incubating and Rearing groups had significantly lower expression compared to layingbirds or no expression at all. As both vasotocin and the BnSTl have been implicated in the regulation of stress, this suggests a possible attenuation of the stress response in incubating hens. However, as the stress response was not measured, this hypothesis needs to be tested in future studies. No differences in expression were found between groups in the BnSTm, SON or POM for either peptide. These results complement previous data on the role of mesotocin in chick rearing and suggest for the first time that the avian BnSTl may be involved in the control of maternal behaviour, possibly through the attenuation of the stress response and/or through playing a role in diminishing social interaction in incubating hens.

4.1 Introduction

4.1.1 Roles of nonapeptides in maternal behaviour

The importance of nonapeptides for maternal behaviour has already been touched upon in Chapter 1 but more detailed examples are provided in this section.

4.1.1.1 Role of oxytocin in maternal care in mammals

Variations in maternal care are associated with differences in oxytocin receptor levels in rats as rats which consistently exhibited more arch-back nursing were found to have higher levels of receptor binding in the central amygdala compared to rats which nursed less (Fancis et al., 2000). In lactating rats, binding to both the OTR and the V1a receptor in the BnST and binding to OTR in the MPOA was higher than in non-lactating dams (Bosch et al., 2010). In human mothers, plasma oxytocin concentrations increased 3 to 10 minutes before suckling in response to stimuli from the baby indicating feeding time was approaching or in response to the mother preparing for a scheduled feeding session. Both oxytocin and prolactin were also released in response to suckling itself (Barofsky et al., 1983a). As mentioned in Chapter 1, oxytocin is capable of inducing maternal care in virgin animals as illustrated by a study in rats where its administration let to the display of full maternal behaviour in ovariectomised virgin female rats which had been primed with estrogen 48 hours prior (Pedersen et al., 1982). Additionally, in naked mole rats only the queen gives birth, but most rats take care of pups and this may be reflected in the distribution of oxytocin in the brain as this

species showed high levels of oxytocin immunoreactivity in the MPOA and the nucleus accumbens, both of which have been implicated in maternal behaviour (Rosen et al., 2008).

4.1.1.2 Role of vasopressin in maternal care in mammals

The role of vasopressin is also very important, and this peptide is often involved in the control of specific aspects of maternal care, such as maternal aggression. Blocking V1a receptors in the central amygdala of rats showing high anxiety and high maternal care/aggression lowered maternal aggression, while administration of synthetic vasopressin to this region in rats bred for lower anxiety/maternal care elevated their naturally low levels of maternal aggression (Bosch and Neumann, 2010). However, vasopressin can also facilitate general maternal behaviour, as demonstrated in chronically stressed rats where chronic administration of vasopressin to the right lateral ventricle increased overall maternal care, including nursing, grooming and pup retrieval (Coverdill, 2012). As already noted above, the V1a vasopressin receptor is often involved in the control of maternal behaviour. Central administration of V1a receptor antagonists in the PVN of lactating rats impaired nursing and mother-pup interaction (Bayerl et al., 2014), while upregulation of the V1a receptor as well as central administration of vasopressin improved maternal care (Bosch and Neumann, 2008).

4.1.1.3 Nonapeptides and maternal care in birds

In zebra finches, there was higher Fos-immunoreactivity in mesotocin neurones in nest-building birds of both sexes, and in vasotocin neurones in

relation to the amount of nest-building material picked and time spent with the partner in the nest, showing that both peptides may be involved in nest-building (Hall et al., 2015). Another study in this species showed that peripheral but not central infusions of oxytocin receptor antagonists strongly reduced nest-building behaviour in females but not males, while peripheral injections of V1a receptor antagonists had much smaller effects in both sexes, suggesting that the effects of mesotocin on nesting was regulated peripherally, most likely through the oviduct, as injections closer to that area had stronger effects than injections into the breast muscle (Klatt and Goodson, 2013b). Similar to the study on marmoset monkeys cited previously in this thesis (Taylor and French, 2015), oxytocin receptor antagonists do not cross the blood brain barrier and in this case the change in behaviour was not caused when they were administered centrally, suggesting that they may act on receptors in the oviduct which then signal to neurones of the brain behaviour network, eliciting a change in behaviour. Mesotocin appears to be necessary for maternal care in the Thai hen, as mesotocin-immunoreactive neurones were found to grow in number throughout the reproductive cycle from non-laying to rearing hens in several areas involved in maternal behaviour including the PVN, POM and SOv. In a study by Chokchaloemwong *et al* (2013), SOv meotocin neurones peaked significantly in number during incubation but decreased to the same levels as in laying hens by the 14th day of rearing. In the same study, POM and PVN neurones were significantly greater in number in incubating and rearing compared to non-laying hens, and PVN neurones were also greater in number in rearing compared to laying hens (Chokchaloemwong et al., 2013). As mentioned previously, evidence for the importance of this peptide was also found in the turkey. More specifically, the expression of c-fos in the POM, PVN and BnSTm was significantly higher in turkey hens rearing chicks than in non-rearing hens and mesotocin antagonists completely abolished rearing behaviour in this species (Thayananuphat et al., 2011).

4.1.2 Aim and hypothesis

The aim of this study was to characterise the natural changes in mesotocin and vasotocin mRNA expression throughout the reproductive cycle in the domestic hen, to provide further evidence for the role of mesotocin in maternal care, and to test the hypothesis that vasotocin may also be involved in this behaviour, similarly to its mammalian orthologue vasopressin. In addition to nuclei in the brain which have been well-established as relevant to maternal behaviour, the aim was to examine nonapeptide expression in the lateral division of the BnST which has not been studied in this context, although the medial division of the nucleus is known to be important for maternal care.

Testosterone was shown in Chapter 3 to induce mesotocin mRNA expression in the PVN of female chickens. However, in the context of maternal care, even though it was predicted that higher mesotocin mRNA expression would be observed in the PVN of incubating and chick-rearing birds, compared to layers, testosterone was not expected to be responsible for this, as its plasma levels have been shown in previous studies to decrease with the onset of incubation. In the BnSTl however, nonapeptide mRNA expression was also shown to be induced by testosterone in Chapter 3, and the changes of nonapeptide expression in this brain area throughout the reproductive cycle had not been characterised. Plasma levels of testosterone were measured in order to confirm that it was not responsible for any changes in nonapeptide mRNA expression in the PVN during the maternal period in the chicken, and in order to find out whether changes in its plasma levels correlated to any changes nonapeptide mRNA expression in the BnSTl.

Hypothesis:

Based on the previously cited mammalian literature on the involvement of oxytocin and vasopressin in maternal behaviour, as well as recent evidence for the involvement of mesotocin in chick rearing, it was hypothesised that both mesotocin and vasotocin mRNA expression would be higher in brain areas related to maternal behaviour including the PVN, BnST, POM and SON of incubating and rearing hens compared to laying hens. Based on previous studies, plasma levels of testosterone were expected to decrease with the onset of incubation and remain lower throughout incubation compared to laying.

4.2 Materials and methods

Details of the materials used in this chapter as well as the in situ *hybridisation protocol used can be found in Chapter 2. What follows is a description of the procedures specific to this chapter.*

4.2.1 Animals and housing

Adult female chickens (*Gallus gallus*) from the F1 of a cross produced at the Roslin Institute between pure Silkie and White Leghorn lines were housed in 2 square meter floor pens under standard lighting conditions (14L:10D, lights on at 6am). The birds were divided into four groups (n=8) – Laying (laying

status confirmed post mortem), Onset of incubation (taken as the third day of continuously sitting on eggs), Incubating (hens which had been sitting on eggs for two weeks) and Rearing (hens which had been rearing chicks for 24 hours). Both groups of incubating hens (Onset and Incubating) and the rearing hens were housed in a quiet experimental room equipped with video cameras in floor pens split in two with a low barrier which the hens could jump over. Each side of the pen was equipped with a nest box (30x50x28 cm) filled with ten fertile chicken eggs in order to encourage them to incubate. Food and water were provided *ad libitum*. Unhatched embryos were decapitated following disruption of the amniotic membrane. All animals were treated in accordance to the Home Office Guidelines and the Roslin Institute Animal Welfare protocols.

4.2.2 Determining the onset of incubation

The onset of incubation was determined with the help of video recordings and a simple behavioural test. The video recording assisted in confirming the amount of time a hen had been spending in the nest. In addition to that, once a hen had been sitting for two days, an experimenter entered the pen, approached and touched the animal. If the hen continued to sit on the eggs it was assumed that incubation had truly begun while if the bird left the nest it was not yet considered to be incubating.

4.2.3 Tissue collection and sectioning

On the morning of day 3 of incubation, day 14 of incubation, or on the morning after the chicks had hatched, hens were euthanised with an

overdose of anaesthetic. A laying hen was euthanised at the same time as each rearing hen and each bird's reproductive status was confirmed post mortem by examining the ovaries. Brains were collected immediately after death, frozen on dry ice and stored at -70˚ C. Coronal brain sections were cut on a cryostat at 15µm in four series so that every two sequential sections on a slide were 45µm apart. Sections were mounted on polysine slides and stored at -70˚C.

4.2.4 Enzyme-linked Immunosorbent Assay (ELISA) to measure plasma testosterone

4.2.4.1 Optimisation of testosterone assay

In order to determine what concentration of sample gave optimal results in terms of sensitivity and plasma interference, the assay was first optimised.

Testosterone ELISA Kit was used in this procedure, following the manufacturer's protocol. Chicken serum was stripped with 1% charcoal with 0.1% dextran sulphate and spiked with 500pg/ml testosterone standard from ELISA Kit. This was diluted in buffer spiked with the same amount of testosterone to a concentration of 1:10, 1:20 and 1:40. 1% Steroid displacing reagent was used per sample. Along with this spiked serum, one sample from a laying, one sample from an incubating hen, one sample from a juvenile chick not injected with a steroid and one sample from a juvenile chick injected with testosterone were run in the three concentrations above to verify that the assay was working and decide the best concentration. After the optimisation it was decided that a concentration of 1:20 was optimal and this concentration was used in the subsequent assay.

4.2.4.2 Detection of testosterone in chicken plasma

The assay was performed according to manufacturer's protocol. Each sample and standard were run in duplicate. As only the difference beween groups and not the actual concentration of testosterone was of interest, high, medium and low controls for drift (differences in pipetting between earlier and later wells) at the beginning and end of the plate were not included but in order to avoid possible drift influencing the comparison between groups, samples were processed in order such that one sample from each of the four groups was present every four samples, thus ensuring that if drift was present it would have affected the four groups equally. The intra-assay variability (coefficient of variation) was %CV=1.692.
Figure 4.1 shows an example standard curve for the assay (four-parameter logarythmic curve).

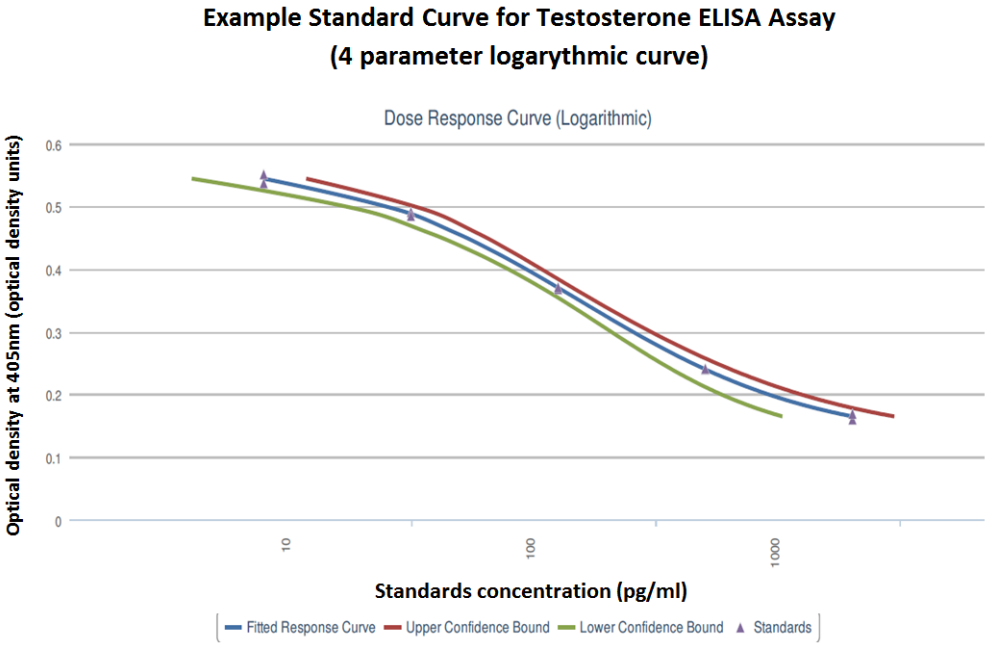

Figure 4.1 Example standard curve (4-parameter logarythmic curve) for testosterone ELISA assay.
The fitted response curve can be seen in blue with the upper confidence interval in red and the lower confidence interval in green.

Assay buffer 3 stock was diluted 1:10 just before use in MilliQ water. The testosterone standard was allowed to warm up to room temperature and was then diluted in 1x buffer 3 to the following concentrations:

2 000, 500, 125, 31.25 and 7.81 pg/ml. Diluted standards were used within 60 min of preparing. Wash buffer was prepared by diluting 5ml stock in 95ml MilliQ water. All reagents were brought to room temperature for at least 30 minutes prior to opening. All standards and samples were run in duplicate.

150 µl Assay Buffer 3 were pipetted into NSB and 100µl into the B_0 (0 pg/ml Standard) wells. 100 µl of Standards 1 through 5 and samples to be measured were pipetted into the appropriate wells. 50 µl of Testosterone ELISA Antibody (cat. # 80-0433) were added to each well, except the Blank,

TA (total activity) and NSB (nonspecific binding) wells. The plate was incubated at room temperature on a plate shaker for 1 hour at 500 rpm. 50 µl of Testosterone ELISA Conjugate (cat. # 80-0432) was added to each well, except the Total Activity (TA) and Blank wells. The plate was incubated at room temperature on a plate shaker for 1 hour at 500 rpm. The content of the wells was pipetted out and they were washed three times by adding 400 µl of wash solution to every well. After this, the wells were emptied as before, and the plate was turned upside down and tapped on a lint free paper towel to remove any remaining wash buffer. 5 µl blue Conjugate were added to the TA wells and 200 µl of the pNpp Substrate solution were added to every well. The plate was incubated at 37°C for 1 hour sealed with a plate sealer provided. 50 µl Stop Solution were added to every well. The optical density was read on a microplate reader at 405nm.

4.2.5 Designing oligonucleotide probes

The process of designing the probes used in this procedure is described in Chapter 2 and the probes used for vasotocin and mesotocin ISH were the same as used in Chapter 3. For nucleotide composition and photos of negative controls, see the Materials and Methods section in Chapter 3.

4.2.6 Radioactive *in situ* hybridisation (ISH)

The same protocol as in Chapter 3 was used in this study. The same probes as in Chapter 3 were used as well. The protocol is described in detail in Chapter 2. As before, at the end of the protocol, slides were rinsed briefly in

ddH_2O and allowed to air dry overnight. After this, they were dipped in Ilford K5 Gel Emulsion under safe light conditions and exposed for 17 days at 4˚C.

4.2.7 Image capture and analysis

Slides were coded so that the experimenter analysing them was unaware of the identity of each chicken. Photographs of the regions of interest (ROI) on both the left and right side of the brain were taken on a Nikon E400 (Nikon Co., Ltd) microscope with an Axiocam 105 colour camera and Zen image capture software (Carl Zeiss Ltd). Images for analysis were captured at x4 magnification for the PVN and x1 magnification for the POM, SON and BnSTm. mRNA signal for the BnSTl was weak and was measured by counting individual neurones containing silver grains under the microscope at x10 and x20 magnification. For the PVN, BnSTm and POM and SON, the mRNA signal density in the region of interest in % area covered by signal (equivalent to mm^2/mm^2) was measured with the Image J image-processing software by drawing around the region and applying a threshold which only picked up pixels above certain intensity in order to reduce background. A background measurement from an adjacent area was also taken from each image in % area covered by signal (equivalent to mm^2/mm^2) and subsequently subtracted from the ROI measurement to eliminate the effect of different background levels on the accuracy of the final value. Signal in the POM and SON was measured at the level of the anterior commissure (CA) in 4-6 hemi-sections per bird. Signal in the PVN and BnST (both lateral and medial) was measured in 10-12 hemi-sections per bird caudal to the first appearance of the CA.

4.2.8 Statistical analysis

The Sigmaplot (Sigmaplot Software Inc) statistical software was used for all statistical analysis. One-way ANOVA and Tukey's post-hoc test or Kruskal-Wallis ANOVA on ranks and Dunn's post-hoc test when the assumptions for parametric ANOVA were not met, were performed on the data using treatment as the independent variable. $P<0.05$ was considered statistically significant. An average of all measurements from the left- and right-hand side of the brain was used in the analysis. Normality test used was Shapiro-Wilk.

4.3 Results

4.3.1 Incubating and rearing behaviour

All incubating birds in the experiment spent nearly all of their time in the nesting box with only occasional pauses to feed or drink. They showed very little interaction with the other bird in the pen and did not show any obvious response to the experimenter entering the pen. Surprisingly, they also did not show any aggressive behaviour when approached while sitting on the nest. All birds in the rearing group hatched chicks although the number of chicks hatched varied between 1 and 10. Six out of the eight rearing birds remained in the nest with their chicks until they were removed for tissue collection and only two out of the eight were out of the nest and moving around the pen with their chicks at the end of the experiment. However, all of the rearing birds were observed covering the chicks with their wings and producing maternal vocalisations.

4.3.2 Plasma testosterone concentration throughout the reproductive cycle

Plasma concentrations of testosterone were not detectable in the majority of samples for the three non-laying groups, while testosterone was detected in the plasma of all layers (average concentration of 1.16ng/ml). Because of this, no statistical analysis could be performed on the data but the lack of detectable testosterone in the vast majority non-laying birds indicated that testosterone decreased significantly with the onset of incubation.

4.3.3 Distribution of nonapeptide mRNA

The distribution of mesotocin and vasotocin mRNA-expressing neurones in the brain of adult hens followed the same pattern as that in juvenile hens described in Chapter 3. Diagrams and photomicrographs for both peptides are shown on Figure 4.2 (A, B and C).

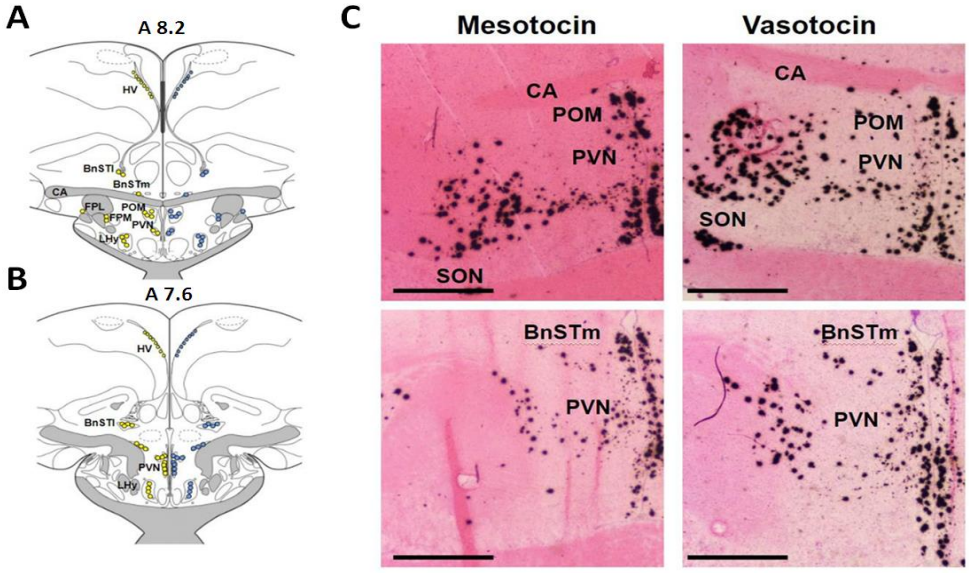

Figure 4.2 Distribution of Mesotocin and Vasotocin mRNA expression in the hypothalamus of the juvenile hen.
A and B. Schematic diagrams of coronal sections illustrating the distribution of mesotocin (yellow dots) and vasotocin (blue dots) mRNA-expressing neurons in the brain of hens (the same as in Chapter 3). Figures adapted from the Stereotaxic Atlas of the Brain of the Chick (Kuenzel and Masson, 1988), coordinates given at the top of each image, where 'A' followed by a number signifies a brain plate the given number of millimetres anterior to a zero reference plane.
C. Photomicrographs showing mRNA expression (black spots) for mesotocin and vasotocin at coordinates in the brain corresponding to the diagrams. Scale bars = 1000μm. The two peptides were present in the same brain areas. LHy, Regio lateralis hypothalami (lateral hypothalamic area); PVN, nucleus paraventricularis magnocellularis; V3, ventriculus tertius (third ventricle), anterior commissure (CA), POM (medial preoptic area), SON (supraoptic nucleus, only visible in the top part of image C), lateral and medial forebrain bundle (FPL and FPM), medial bed nucleus of the stria terminalis (BnSTm), lateral bed nucleus of the stria terminalis (BnSTl), hyperstriatum ventrale (HV).

The highest density of expressing neurones for both peptides was detected along the third ventricle in the PVN and POM, as well as the SON and the LHy. Strong signal was also present around the FPL and FPM (lateral and medial forebrain bundle) and in the BnSTm. Signal in the BnSTl was much weaker by comparison but could be observed under higher magnification. Some silver grains were also present in the hyperstriatum ventrale (HV).

4.3.4 Mesotocin mRNA expression throughout the reproductive cycle

Mesotocin mRNA levels were measured in several brain areas for each of the experimental groups via ISH. The areas examined were the medial part of the PVN, the SON, the medial and lateral BnST and the POM. Two separate populations could be observed in the BnSTl - a population directly next to the lateral ventricle (BnSTl1) and a population below the lateral ventricle (BnSTl2, the same population which was examined in the experiment described in Chapter 3). The positions of the two populations in relation to the lateral ventricle and to each other are shown on Figure 4.3. The two populations could not be captured on the same image as BnSTl1 (Figure 4.4) showed stronger signal while silver grains in BnSTl2 could only be seen under very high magnification under which the first population fell outside the field of vision (Figure 4.5 A).

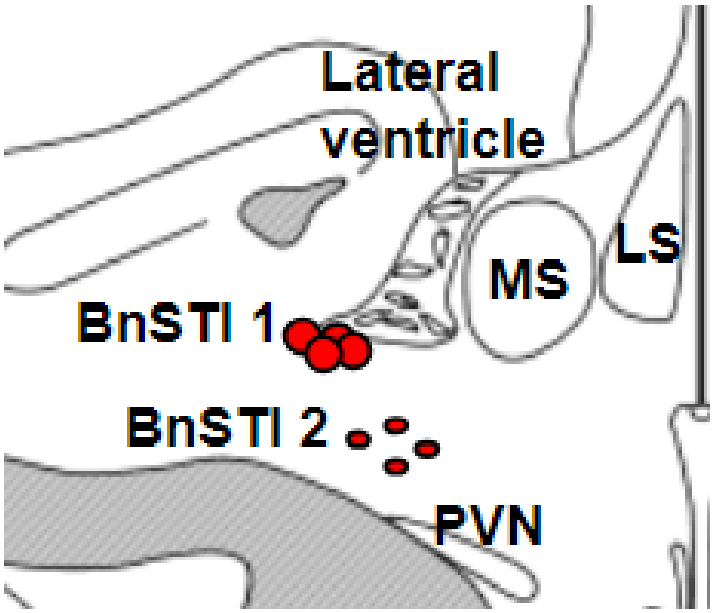

Figure 4.3 Schematic representation of the two population of nonapeptide neurones observed in the BnSTl of laying hens.
The two nonapeptide neurone populations which had the same location for both mesotocin and vasotocin are represented by red dots. The left-hand side lateral ventricle is shown. The population next to the ventricle (BnSTl1) had stronger mRNA expression in laying hens than the population under the ventricle (BnSTl2) but BnSTl1 showed no expression in any group other than Laying while in BnSTl2 some expression was observed in all groups. PVN (paraventricular nucleus); LS (lateral septum); MS (medial septum). These populations are both visible between coordinates A 8.2 and A 7.6 of the stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988).

As in the BnSTl1 silver grains were only present in hens in the Laying group and they were completely absent in all other groups, no statistical analysis was performed for this population but photomicrographs for each group can be seen on Figure 4.4. For the BnSTl2, the x20 objective had to be used in order to properly observe signal. mRNA expression was compared through statistical analysis for this population and statistical results cited in this

chapter for the BnSTl refer to this population. Photomicrographs of mRNA expression in the four groups, along with graphical representation of results are shown on Figure 4.5 A and B.

In all other areas of the brain analysed in this study, mRNA signal was clearly visible at lower magnification (1x and 4x).

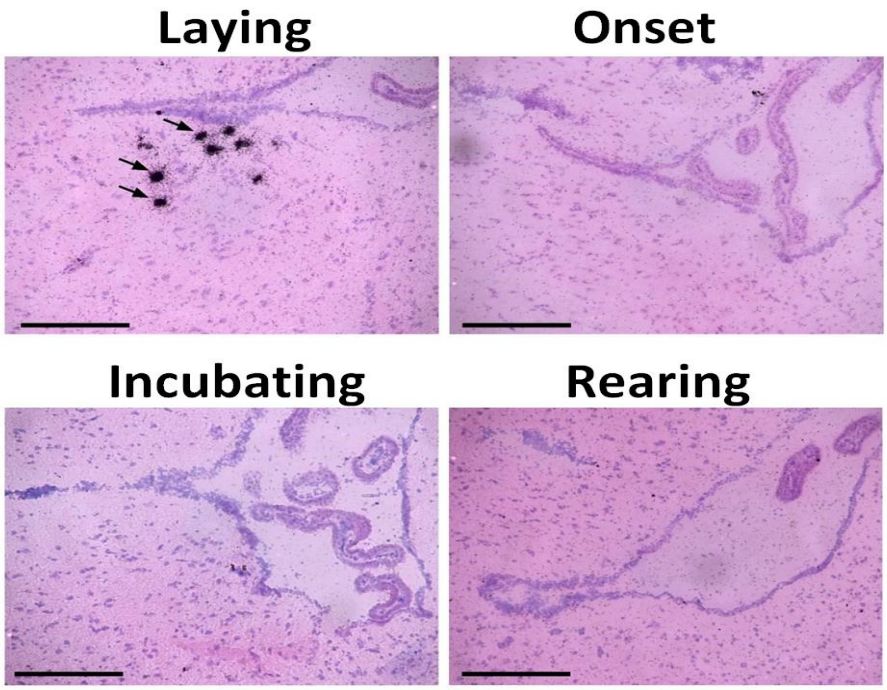

Figure 4.4 Light-field photomicrograph showing mesotocin mRNA expression in the BnSTl population next to the lateral ventricle (BnSTl1) of hens throughout the reproductive cycle. Images show neurons covered by hybridisation signal (silver grains) in the left-hand side upper BnSTl of Laying, Onset, Incubating and Rearing hens. Arrows denote examples of labelled cells. Scale bar = 250μm.

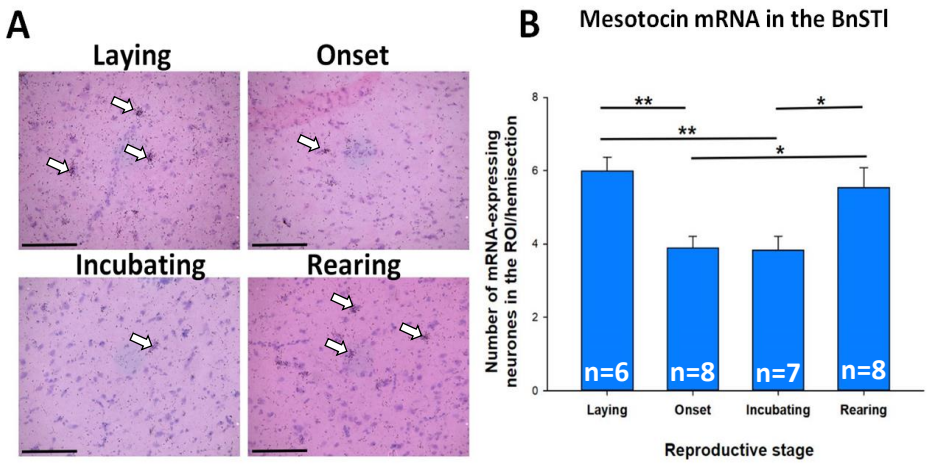

Figure 4.5 Mesotocin mRNA expression in the BnSTl of hens throughout the reproductive cycle.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the left-hand side BnSTl (BnSTl2, under the lateral ventricle) of Laying, Onset, Incubating and Rearing hens. Arrows denote examples of labelled cells. Scale bar = 100μm.
B. There was a significant difference between groups in mesotocin mRNA expression in the BnSTl (One-way ANOVA, $F_{(3,25)}$=6.948, p=0.001). Laying hens had higher expression than Onset (Tukey's test, p=0.009) and Incubating hens (Tukey's test, p=0.010). Rearing hens also had higher expression than both Onset and Incubating hens (Tukey's test, p=0.032 for both comparisons). n=6-8. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal). An average of measurements from the left- and right-hand side of the brain was taken for each bird. * denotes p<0.05, **denotes p</=0.01. Data are presented as mean + SEM.

In the BnSTl (BnSTl2), there was no difference between the Laying and Rearing groups or the Onset and Incubating groups but Laying and Rearing both had significantly higher mRNA expression than Onset and Incubating (p<0.05, Tukey's test). Data are shown on Figure 4.5 B.

mRNA in the PVN showed an increase from Laying to Rearing hens (One-way ANOVA, $F_{(3,21)}$=4.456, p=0.014) with only the difference between Laying and Rearing birds being significant (p=0.008, Tukey's test). Figure 4.6 A and

B shows photomicrographs of mesotocin mRNA expression in the PVN for the four groups, together with graphical representation of results.

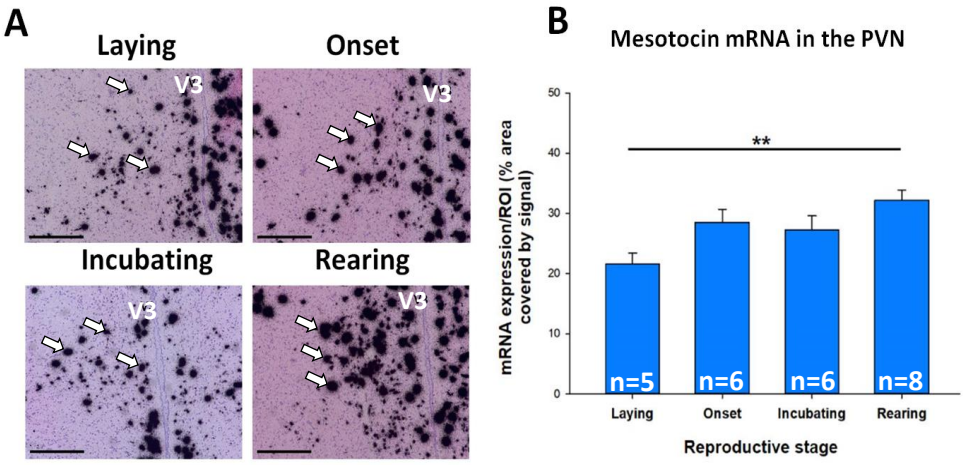

Figure 4.6 Mesotocin mRNA expression in the PVN of hens throughout the reproductive cycle.

A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the left-hand side PVN of Laying, Onset, Incubating and Rearing hens. Arrows denote examples of labelled cells. The third ventricle (V3) is shown. Scale bar = 250µm.

B. Mesotocin mRNA expression in the PVN showed differences between groups (One-way ANOVA, $F_{(3,21)}$=4.456, p=0.014). There was an increase from Laying to Rearing hens with only the difference between Laying and Rearing birds being significant (Tukey's test, p=0.008). n=5-8. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal. In addition, some sections for this brain region were lost due to a mistake during the developing stage.) Data are presented as mean + SEM. **denotes p<0.01.

No difference was found in the SON (One-way ANOVA, $F_{(3,16)}$=0.730, p=0.549)., POM (One-way ANOVA, $F_{(3,16)}$=0.854, p=0.485) or BnSTm (Kruskal-Wallis ANOVA on ranks, $H_{(3)}$=1.572, p=0.666) Figures 4.7 A and B, 4.8 A and B, and 4.9 A and B respectively show photomicrographs of mesotocin mRNA hybridisation signal for these brain regions in the four groups, together with graphical representation of results.

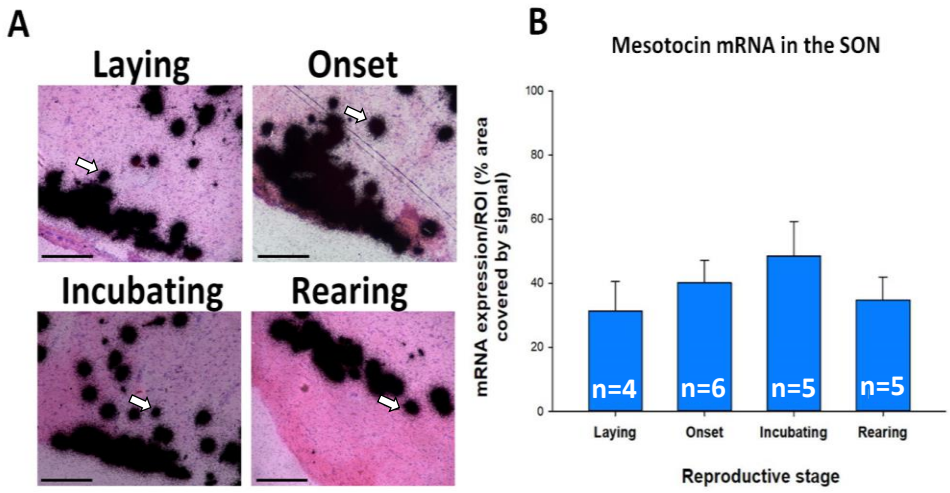

Figure 4.7 Mesotocin mRNA expression in the SON of hens throughout the reproductive cycle.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the left-hand side SON of Laying, Onset, Incubating and Rearing hens. The lower left-hand side edge of each brain section is visible. Arrows denote examples of labelled cells. Scale bar = 250µm.
B. There was no significant difference between groups in mesotocin mRNA expression in the SON (One-way ANOVA, $F_{(3,16)}$=0.730, p=0.549). n=4-6. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal. In addition, some sections for this brain region were lost due to a mistake during the developing stage.) Data are presented as mean + SEM

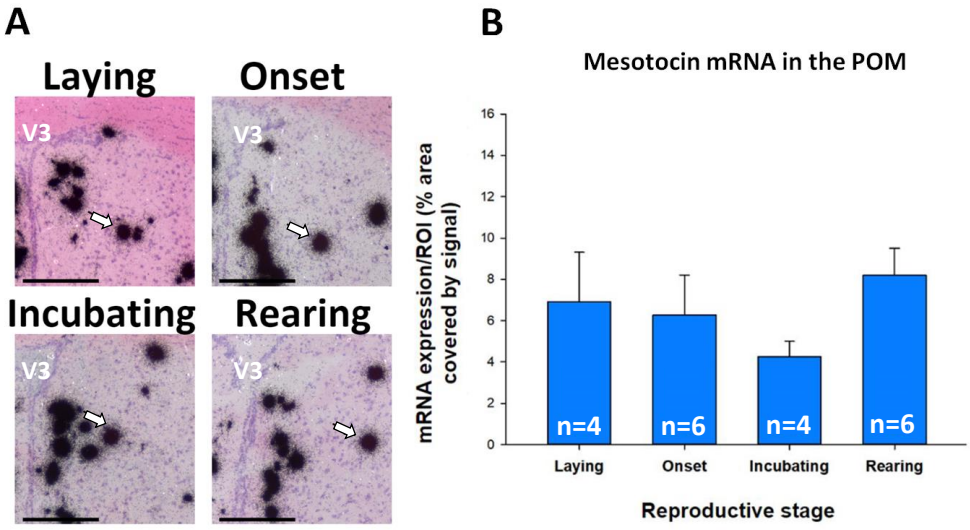

Figure 4.8 Mesotocin mRNA expression in the POM of hens throughout the reproductive cycle.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the right-hand side of the POM of Laying, Onset, Incubating and Rearing hens. The third ventricle (V3) is indicated. Arrows denote examples of labelled cells. Scale bar = 250µm.
B. There was no significant difference between groups in mesotocin mRNA expression in the POM (One-way ANOVA, $F_{(3,16)}$=0.854, p=0.485). n=4-6. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal. In addition, some sections for this brain region were lost due to a mistake during the developing stage.) Data are presented as mean + SEM.

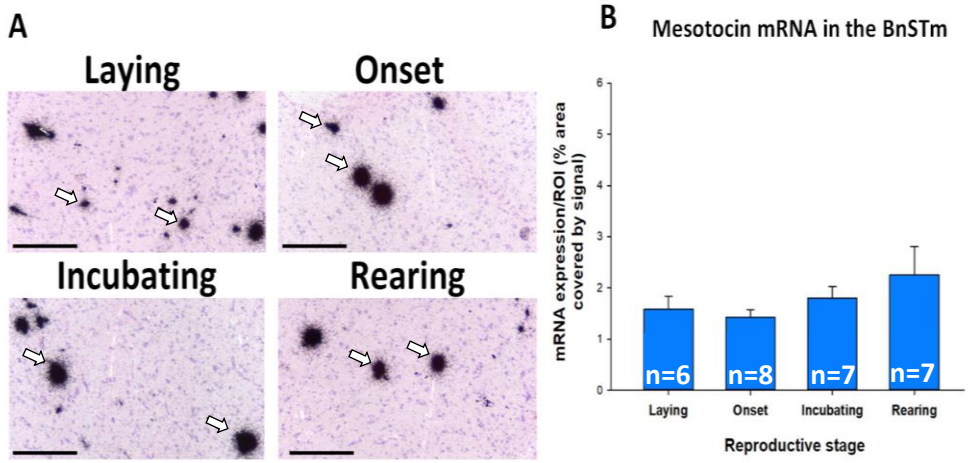

Figure 4.9 Mesotocin mRNA expression in the BnSTm of hens throughout the reproductive cycle.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the left-hand side BnSTm of Laying, Onset, Incubating and Rearing hens. The third ventricle is not visible but is positioned to the right of each image. Arrows denote examples of labelled cells. Scale bar = 250µm.
B. There was no significant difference between groups in mesotocin mRNA expression in the BnSTm (One-way ANOVA, $F_{(3,24)}$=0.946, p=0.434). n=6-8. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal. Data are presented as mean + SEM.

4.3.5 Vasotocin mRNA expression throughout the reproductive cycle

Vasotocin mRNA expression was measured in several brain areas for each of the experimental groups via ISH. The areas examined were the medial part of the PVN, the SON, the medial and lateral BnST and the POM. As with mesotocin, two separate populations could be observed in the BnSTl (BnSTl1 and BnSTl2, positions of both were the same as for mesotocin and are shown on Figure 4.3), one directly next to the lateral ventricle (BnSTl1, Figure 4.10) and one below the ventricle (BnSTl2, Figure 4.11 A). As with mesotocin, in the BnSTl1, silver grains were only present in laying hens and

they were completely absent in all other groups so no statistical analysis was performed for this population. For the BnSTl2, for which expression was quantified, the x20 objective had to be used in order to observe signal. mRNA expression was compared through statistical analysis for this population and statistical results cited below for the BnSTl refer to this population. Results are presented graphically on Figure 4.11 B.
In all other areas of the brain analysed in this study signal was clearly visible at lower magnification (1x and 4x).

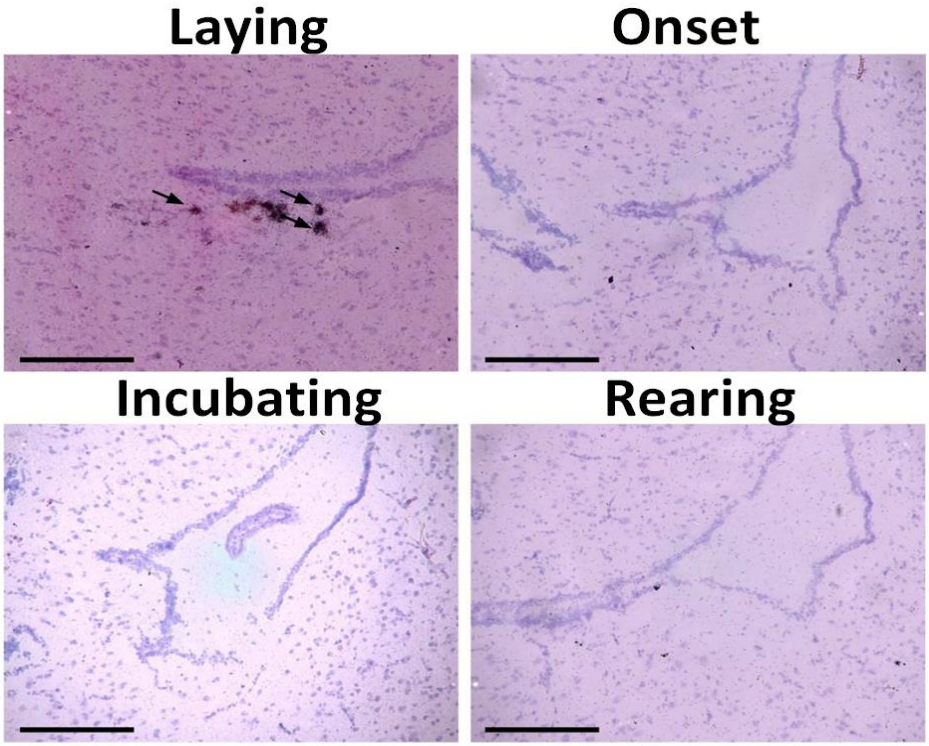

Figure 4.10 Light-field photomicrograph showing vasotocin mRNA expression in the BnSTl population next to the lateral ventricle (BnSTl1) of hens throughout the reproductive cycle. Images show neurons covered by hybridisation signal (silver grains) in the left-hand side upper BnSTl of Laying, Onset, Incubating and Rearing hens. Arrows denote examples of labelled cells. Scale bar = 250µm.

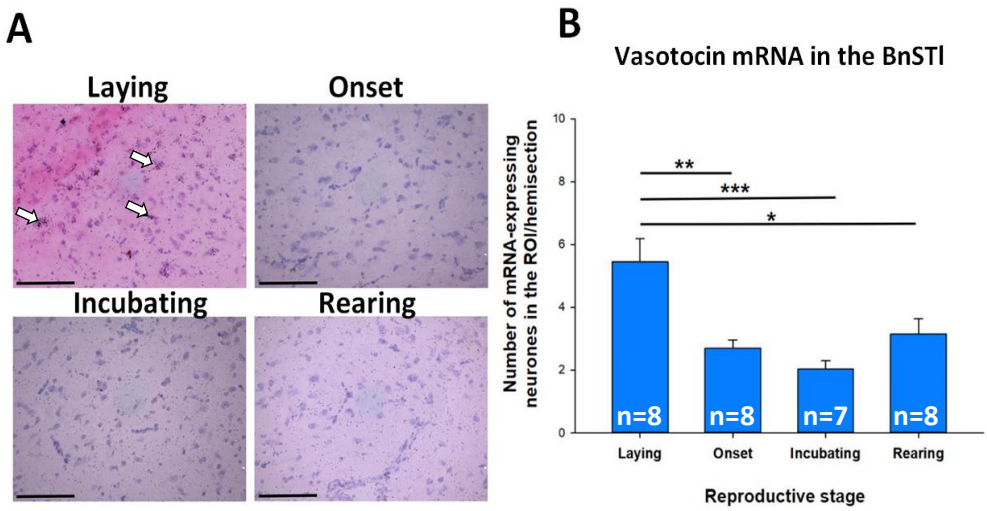

Figure 4.11 Vasotocin mRNA expression in the BnSTl of hens throughout the reproductive cycle.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the left-hand side PVN of Laying, Onset, Incubating and Rearing hens. Arrows denote examples of labelled cells. Scale bar = 250µm.
B. There was a significant difference between groups in vasotocin mRNA expression in the BnSTl (BnSTl2, under the lateral ventricle) (One-way ANOVA, $F_{(3,27)}$=9.160, p<0.001). Laying hens had higher expression compared to Onset (Tukey's test, p=0.002), Incubating hens (Tukey's test, p<0.001) and Rearing hens (Tukey's test, p=0.012). n=7-8. (8 birds per group were included in the initial procedure but one bird was excluded from analysis due to sections being damaged in this area of the brain.) Data are presented as mean +SEM.

In the BnSTl (BnSTl2), One-way ANOVA showed a difference between groups ($F_{(3,27)}$=9.160, p<0.001) and mRNA expression was lower in all three non-laying groups compared to layers (p</=0.01, Tukey's test).
No difference was found between groups in the PVN (One-way ANOVA, $F_{(3,26)}$=2.387, p=0.092). Figure 2.12 A and B shows photomicrographs of vasotocin mRNA expression in this brain area for all groups, together with graphical representation of results.

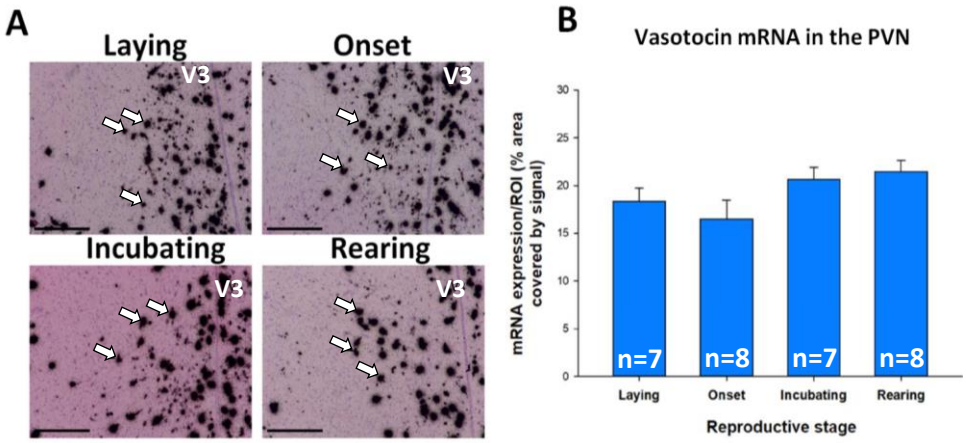

Figure 4.12 Vasotocin mRNA expression in the PVN of hens throughout the reproductive cycle.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the left-hand side PVN of Laying, Onset, Incubating and Rearing hens. Arrows denote examples of labelled cells. The third ventricle (V3) is shown. Scale bar = 250µm.
B. No difference was found between groups for vasotocin mRNA expression (One-way ANOVA, $F_{(3,26)}$=2.387, p=0.092). n=7-8. (8 birds per group were included in the initial procedure but two birds were excluded from analysis due to damage to sections in this brain area. Data are presented as mean + SEM.

The were also no significant differences found in the SON (One-way ANOVA, $F_{(3,19)}$=0.816, p=0.501), POM (One-way ANOVA, $F_{(3,17)}$=2.094, p=0.139) or BnSTm (Kruskal-Wallis ANOVA on ranks, $H_{(3)}$=0.244, p=0.970). Figures 4.13 A and B, 4.14 A and B and 4.15 A and B respectively, show photomicrographs of vasotocin mRNA signal for all four groups together with graphical representation of results.

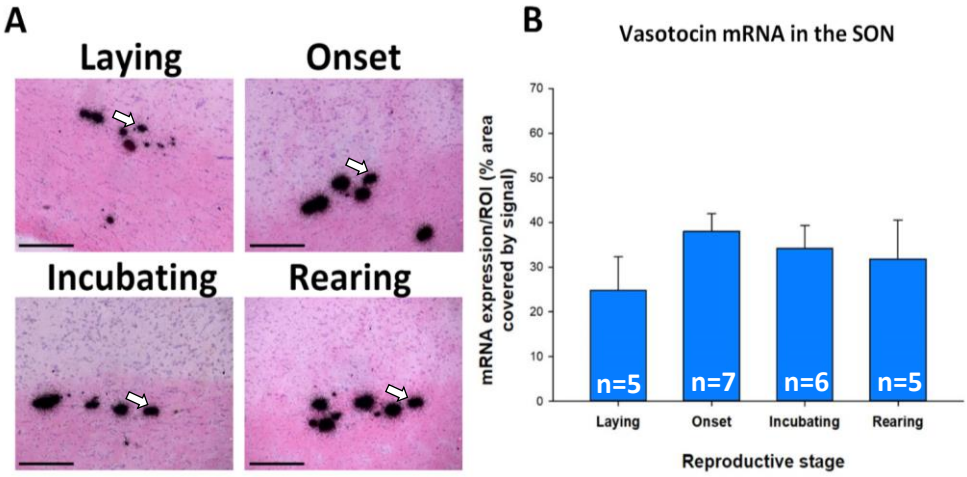

Figure 4.13 Vasotocin mRNA expression in the SON of hens throughout the reproductive cycle.

A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the left-hand side SON of Laying, Onset, Incubating and Rearing hens. The left-hand side lower edge of the brain section is not visible on all images but is positioned directly below the images. Arrows denote examples of labelled cells. Scale bar = 250µm.

B. There was no significant difference between groups in vasotocin mRNA expression in the SON ($F_{(3,19)}$=0.816, p=0.501. n=5-7. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal.) Data are presented as mean + SEM.

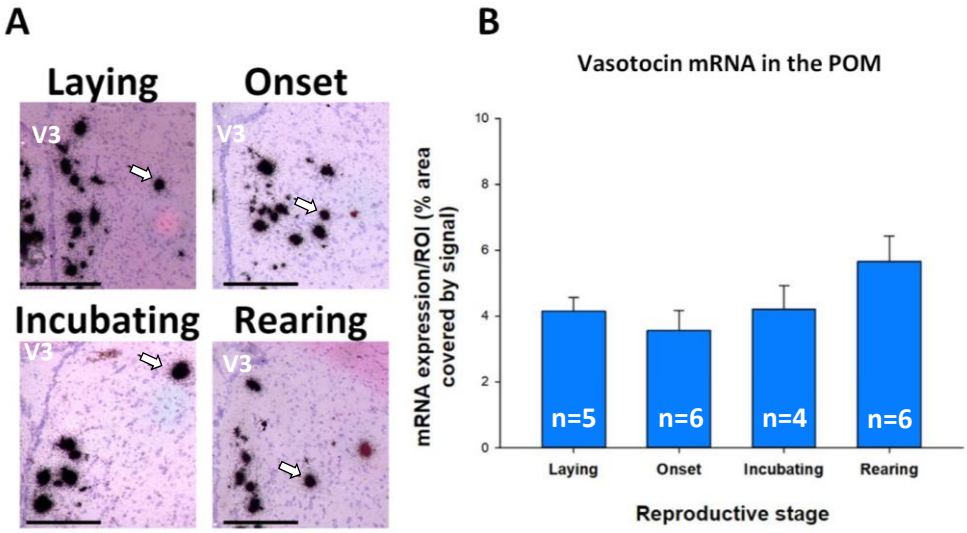

Figure 4.14 Vasotocin mRNA expression in the POM of hens throughout the reproductive cycle.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the right-hand side POM of Laying, Onset, Incubating and Rearing hens. The third ventricle (V3) is shown. Arrows denote examples of labelled cells. Scale bar = 250µm.
B. There was no significant difference between groups in vasotocin mRNA expression in the POM ($F_{(3,17)}$=2.094, p=0.139). n=4-6. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal or, in the case of two birds, due to slides being accidentally damaged after the procedure but before they could be analysed.) Data are presented as mean + SEM.

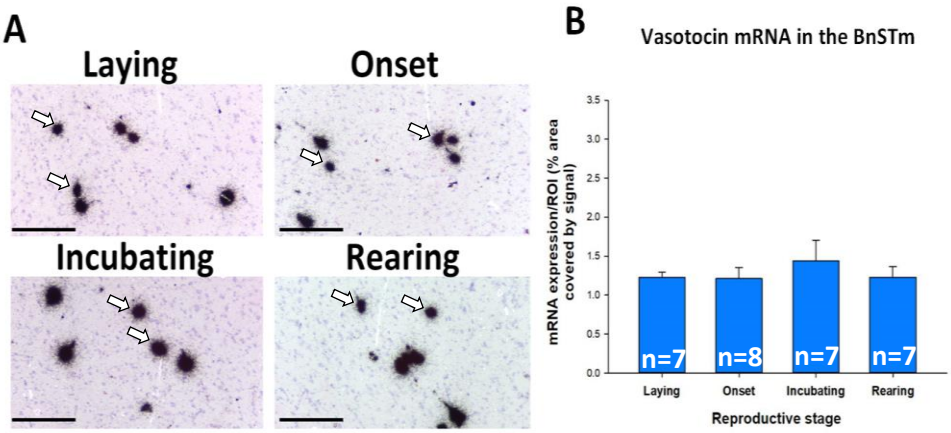

Figure 4.15 Vasotocin mRNA expression in the BnSTm of hens throughout the reproductive cycle.
A. Photomicrographs show neurons covered by hybridisation signal (silver grains) in the BnSTm of Laying, Onset, Incubating and Rearing hens. The third ventricle is not visible but is positioned to the right of each image. Arrows denote examples of labelled cells. Scale bar = 250µm.
B. There was no significant difference between groups in vasotocin mRNA expression in the BnSTm (One-way ANOVA, $F_{(3,25)}$=0.279, p=0.842). n=7-8. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to dirt or damage to sections obscuring the signal.) Data are presented as mean + SEM.

4.4 Discussion

4.4.1 Plasma testosterone concentration throughout the reproductive cycle

The presence of detectable testosterone in the plasma of laying hens, and the fact that it was not possible to detect it with the ELISA assay used in this study in the majority of samples from the other three groups, is in agreement with previous studies in birds which have demonstrated that testosterone levels are lower when parental behaviours are taking place and reproduction

is downregulated (Hall, 1986; Richard-Yris et al., 1987). Even though it was demonstrated in Chapter 3 that testosterone could cause an increase in mesotocin mRNA expression in the PVN of juvenile female chickens, as predicted, it does not seem to be responsible for the increase in mesotocin expression related to maternal behaviour, as the highest expression of mesotocin mRNA in this brain region was observed at a time when testosterone was undetectable in plasma. It was also shown in Chapter 3 that testosterone could increase both mesotocin and vasotocin in the BnSTl. This was once again demonstrated in juvenile chickens rather than adults, although results were similar to results obtained in a previous study for vasotocin in the BnSTm of adult quail (Aste et al., 2013). A decrease in the mRNA expression of both mesotocin and vasotocin in this brain region seems to occur at the same time as the absence of detectable plasma testosterone at the onset of incubation in this study. However, this does not necessarily mean that the two are connected. Studies involving administration of testosterone in adult incubating chickens, perhaps with direct delivery to the brain, coupled with measurements of mesotocin and vasotocin expression, are necessary in order to determine whether there is a link. In the case of mesotocin in the lower section of the BnSTl under the ventricle (BnSTL2), which was quantified, there is less likely to be a connection as mesotocin mRNA levels in this part of the BnSTl recovered by the first day of rearing while testosterone remained undetectable in plasma. However, mRNA expression of vasotocin in the BnSTl2 population under the ventricle, and of both mesotocin and vasotocin in the BnSTl1 population next to the ventricle, remained lower than in layers (in the BnSTl2) or undetectable (In the BnSTL1), together with testosterone being undetectable in plasma. This allows for the possibility that the presence of testosterone may be a factor affecting mesotocin and vasotocin expression in these cells, although, once again, further studies are necessary to test this hypothesis.

4.4.2 Mesotocin and maternal behaviour

Chokchaloemwong *et al* (2013) found a difference in the number of mesotocin-immunoreactive neurones in the PVN of laying and incubating Thai hens as early as day 10 of incubation, while in the present study the difference in mRNA levels in this nucleus between laying hens and hens on day 14 of incubation had not yet reached significance. It is possible that this discrepancy is due to differences in the breed of bird, the experimental design, and the methods of analysis used in the two studies. Similarly, the present study found no significant difference in mesotocin mRNA expression in the POM between laying and rearing birds, while a difference in number of mesotocin-immunoreactive neurones in this area was reported in Thai chickens (Chokchaloemwong et al., 2013). However, the rearing group in that study had been rearing chicks for 14 days. It is possible that the POM is less involved in the immediate maternal responses after hatch. It may be beneficial to observe how changes in mRNA expression correspond to immunoreactivity across the reproductive cycle, including a wider range of time points. Data from turkeys also showed that, in addition to the PVN, the POM and the BnSTm were among the areas in which c-fos mRNA expression was significantly increased during poult stimulation (the introduction of chicks to females at the end of incubation) (Thayananuphat et al., 2011). However, c-fos only co-localised with a small number of mesotocin-immunoreactive neurones in the BnSTm and none in the POM, suggesting that mesotocin neurones were not responsible for the involvement of these regions in the immediate response to poults. The majority of c-fos expression was observed in unidentified cells in those two nuclei. Identification of these cells as well as studies on c-fos expression during chick stimulation in other species may aid in better understanding the regulation of rearing behaviour in precocial birds and the mechanisms of involvement of specific brain areas. In general, the results presented in this

chapter confirm previous findings in the chicken and turkey which suggested that the mesotocin system in the PVN plays a role in regulating rearing behaviour in precocial birds.

4.4.3 Mesotocin in the bed nucleus of the stria terminalis

Mesotocin mRNA expression was significantly lower during incubation than during egg-laying and chick-rearing (with regards to chick rearing, this was only true for the BnSTl2 population), while vasotocin mRNA expression in this brain region was significantly lower in both incubating and rearing birds compared to layers. The BnST in general has been shown to play a role in sexual behaviours, social interaction, and social recognition in both mammals and birds, but these studies have usually focussed on the medial part of the nucleus, while the BnSTl has been implicated in aggression and stress (Nagarajan et al., 2014). It is unclear whether the BnSTl specifically also has a function in social behaviour. The significant decrease in mesotocin mRNA expression during incubation observed in this study corresponds to the time hens were spending time alone in the nest, likely with limited social interaction, although this was not formally measured. It was observed that mesotocin mRNA expression in the BnSTl2 had recovered on the first day after hatch when tactile and auditory social cues would have been provided by the chicks. If social interaction in the chicken is indeed decreased during incubation, these results would suggest a possible role for the BnSTl in social interaction not directly related to maternal behaviour. It is important to note that expression in this area was very low, and although significant, the differences observed were small – only 2-3 mRNA-expressing neurones difference between groups. This makes some of the conclusions made here regarding events at hatch tentative, as the difference may not necessasily have biological significance, and the recovery was only observed in the

BnSTl2. However, with regards to the changes in mesotocin mRNA expression at the onset of incubation, althought the two BnSTl populations could not be analysed together, both the statistical analysis of the BnSTl2 and the complete lack of signal in non-layers in the BnSTl1 point to a significant decrease. Taking into account the relatively strong expression from neurones in the BnSTl1 of layers, compared to no expression in all other groups, the possible behavioural effects of these changes are worth investigating further. Both mesotocin and its mammalian orthologue oxytocin are also known to be involved in stress and aggression with high levels of these peptides being correlated with reduced stress and increased aggressive behaviours (Goodson et al., 2015; Nephew et al., 2009). As mentioned above, the BnSTl has also been implicated in stress and aggression and incubating chickens are generally reported to be aggressive. Therefore, the expectation was to see an increase in BnSTl mesotocin mRNA during incubation, while in fact the opposite was observed. It is interesting to note that, while aggression was not formally tested for, no aggressive behaviour was observed when entering the pens and approaching the incubating birds in this study. It may be of interest to measure aggression in tandem with mesotocin levels in incubating chickens. However, as the exact relationship between mesotocin, aggression and the BnSTl has not been determined, it is possible that the mesotocin neurones observed in this study are simply not involved in the control of aggression.

4.4.4 Vasotocin in the bed nucleus of the stria terminalis

Data on the involvement of vasotocin in parental care not only in birds but in other egg-laying species is extremely limited. One study in Puerto Rican coqui frogs (in which males care for the eggs) discovered that vasotocin activated aggressive calls during paternal behaviour (Ten Eyck and ul Haq,

2012). A more recent study, once again in frogs, surprisingly found that intraperitoneal injections of vasotocin reduced intensive egg-caring behaviours in males (Schulte and Summers, 2017). However, although in that study there appeared to be a trend in the same direction for females, results for this sex did not reach significance. Therefore, the present study is one of the first to suggest that changes in the vasotocin sytem may play a role in parental care in egg-laying animals.

Vasotocin mRNA in the BnSTl was lower in all other groups in this study, compared with the Laying group. In the population of cells next to the lateral ventricle in particular, the difference was very easy to observe, as no mRNA expression at all could be seen in any non-laying birds.
As already estrablished, the vasotocin system is connected to stress in birds (see Chapter 1) and higher c-fos expression has been recorded in the BnSTl of stressed chickens (Nagarajan et al., 2014). In addition, in some bird species, the stress response is downregulated when efforts are directed towards preserving the progeny (Calisi et al., 2008; Krause et al., 2016). Therefore, it can be proposed that the decrease of vasotocin mRNA during both incubation and rearing observed in both populations of the BnSTl (undetectable in the BnSTl1 and significantly lower in the BnSTl2) compared to egg-laying in the hens in this study may be connected to an attenuation of the stress response in favour of focussing efforts on maternal care.

Conclusion

The present study examined mesotocin and vasotocin mRNA expression in several brain areas reported to be important for maternal behaviour and other social behaviours throughout the reproductive cycle. Greater mesotocin mRNA expression was recorded in the PVN on the first day of rearing compared to laying. This finding is in agreement with previous studies in the

chicken and turkey and suggests a role for mesotocin in the care of chicks. Lower mRNA expression was observed for both mesotocin and vasotocin in the BnSTl at the onset of incubation and during incubation. This appeared to recover by the first day of rearing for mesotocinergic BnSTl neurones located under the lateral ventricle, while vasotocin mRNA expression and expression from mesotocinergic neurones next to the lateral ventricle continued to be low in this nucleus in rearing hens. It is proposed here that this lower expression may be related to a possible decrease in social interaction in incubating hens or - in view of the known involvement of vasotocin in stress and the inhibition of the stress axis with parental behaviour observed in previous studies in other birds and mammals - to a possible attenuation of the stress response during incubation in the chicken.

Future studies utilising mesotocin and vasotocin antagonists or other methods for inhibiting the mesotocin and vasotocin systems including knockout or gene silencing are needed to further confirm the role of these peptides in incubation and rearing. Behavioural observations of the effects of these treatments on maternal behaviour will help establish the importance of nonapeptides in avian maternal care. Studies on the activation of nonapeptide neurones with the display of maternal behaviour are also necessary for understanding their action, especially in areas such as the BnSTl which have not been studied in this context. Lesions of this area in laying, incubating and rearing hens followed by behavioural studies on the response to stressors and social stimuli would help determine its exact role in these behaviours. Overall, these findings contribute to our understanding of the neuropeptides controlling maternal care in avian species and for the first time suggest a possible role for the BnSTl during the maternal period in the chicken.

Chapter 5 Changes in Gonadotropin Inhibitory Hormone Throughout the Reproductive Cycle of the Domestic Hen

Abstract

The RFamide gonadotropin inhibitory hormone (GnIH) produced in the hypothalamus is a known inhibitor of the reproductive system in vertebrates. GnIH neurones show changes throughout the reproductive cycle in many species and this peptide has also been implicated in the control of the stress response and feeding. Its actions are often context-dependent and can vary across species. As GnIH downregulates the expression of gonadal steroids it may be necessary for the onset of maternal behaviour. However, while changes in GnIH levels in the brain have been studied in rodents and altricial avian species throughout the entire reproductive cycle, including the immediate aftermath of birth or hatch, such detailed studies have not been conducted on precocial birds. Establishing the importance of GnIH for incubation and rearing is of consequence for the understanding of the neuroendocrine control of maternal behaviour in birds.

In this study, the aim was to investigate the possible roles of GnIH throughout the reproductive cycle in the domestic hen. Brains were collected from hens going through their natural reproductive cycles at four time points - laying eggs, the third day of sitting on the eggs, designated as the onset of incubation, the fourteenth day of incubation and the first day after the hatching of chicks. The numbers of GnIH mRNA-expressing and immunoreactive cells in the brain were quantified for each of the time points in the paraventricular nucleus (PVN) which is the primary site of expression of GnIH. GnIH mRNA-expressing neurones were also quantified in the lateral hypothalamus (LHy), which is the area of the brain suggested to mediate GnIH's effects on feeding. While there was no significant difference in mRNA

expression in either area, the number of GnIH-immunoreactive neurones was significantly higher on day 14 of incubation than in birds laying eggs. These results support the hypothesis that GnIH is responsible for suppressing the reproductive axis during incubation, but it does not seem to play a role after hatch in this species.

5.1 Introduction

5.1.1 Gonadotropin inhibitory hormone and the control of reproduction

As described in Chapter 1, gonadotropin Inhibitory hormone (GnIH, known as RF-related peptide 3 or RFRP-3 in its most studied mammalian form) is an RFamide expressed mainly in cell bodies in the paraventricular nucleus with fibers most often projecting to the median eminence (Tsutsui et al., 2000; Tobari et al., 2010; Fraley et al.,, 2013; Ubuka et al., 2008; Shimizu and Bédécarrats, 2010; Osugi et al., 2004). It was discovered in 2000 in the brain of the quail and it was shown to inhibit gonadotropin-releasing hormone (GnRH) in cultured quail anterior pituitaries (Tsutsui et al., 2000). The presence of a variant of this peptide was later confirmed in the brains of other avian species including the zebra finch (Tobari et al., 2010), the Pekin duck (Fraley et al., 2013), the European starling (Ubuka et al., 2008), the chicken (Shimizu and Bédécarrats, 2010) and the white-crowned sparrow (Osugi et al., 2004). The localisation of GnIH neurones in all four of these species was also in the PVN (Tobari et al., 2010; Fraley et al., 2013; Osugi et al., 2004) but fibres were found not only in the ME but also in the septum, POM and optic tectum in the zebra finch (Tobari et al., 2010) and throughout the diencephalon in the duck (Fraley et al., 2013). Orthologues of GnIH performing similar functions have been confirmed in the brains of a number

of mammalian species including rats (Clarke et al., 2012), mice (Clarke et al., 2012), sheep (Clarke et al., 2012), pigs (Li et al., 2012) and bovines (Tanco et al., 2016), as well as in amphibians (Pinelli, et al., 2015) and fish (Choi et al., 2017; Moussavi et al., 2013, Qi et al., 2013), demonstrating that this peptide has been conserved throughout evolution. Aside from the brain, GnIH as well as its receptor is also expressed in the gonads as demonstrated in European starlings (McGuire et al., 2011) and in the pig (Li et al., 2012). GnIH itself can be affected by gonadal steroids as estrogen and progesterone have been shown to downregulate it in mature chickens (Maddineni et al., 2008). As stated in Chapter 1, the main function of GnIH is as an inhibitor of reproduction.

5.1.2 Seasonal, developmental and sex-related changes in gonadotropin inhibitory hormone

5.1.2.1 Seasonal changes

GnIH shows seasonal changes, and its levels have been found to vary throughout the reproductive cycle in birds, mammals and fish, often influenced by sex and developmental factors (Amorin and Calisi, 2015; Bentley et al., 2003; Calisi et al., 2008; Ciccone et al., 2004a; Dixit et al., 2017; Li et al., 2012; McGuire et al., 2011; Moussavi et al., 2013; Small et al., 2008; Smith et al., 2008; Zhao et al., 2014).

Avian species provide a good model for the the study of seasonal and context-dependent changes in the GnIH system in the brain, and a variety of species have been examined. In the Eurasian tree sparrow, GnIH expression was significantly lower in the breeding season compared to non-breeding season, and birds on short days had higher GnIH expression than birds on

long days (Dixit et al., 2017). In addition, Eurasian tree sparrows kept on long days which had regressed testes, had similar expression levels as short-day birds (Dixit et al., 2017). In house sparrows, neuronal activation in the hypothalamus in response to stress, as determined by expression of the early gene EGR-1, was higher in spring than in autumn, while the number of GnIH-immunoreactive neurones was larger in autumn, and an increase in the number of GnIH-immunoreactive neurones was also seen with stress only in spring (Calisi et al., 2008). In European starlings, not only central expression of GnIH but also GnIH expression in the gonads was found to be controlled seasonally with lower GnIH expression during the breeding season, possibly under the control of melatonin as melatonin receptors are localised in the gonads and GnIH expression is increased by melatonin (McGuire et al., 2011).

GnIH neurones in the avian brain appear to change not only in number but also in size throughout the reproductive cycle. When GnIH neurones were studied in house and song sparrows going through an artificial reproductive period established through photostimulation manipulation, GnIH cell bodies were larger in size in photorefractory birds at the end of the breeding season than in photosensitive and photostimulated sparrows (Bentley et al., 2003). Measurements of the size of GnIH cell bodies in European starling also showed variations throughout the reproductive season with cell body size higher in the middle of the season compared to earlier time points (Amorin and Calisi, 2015). As mentioned before, and most relevant to this study, GnIH expression was found to begreater in the hypothalamus of incubating hens compared to layers, which supports the hypothesis that this RFamide may have a role in the control of incubation (Ciccone et al., 2004a; Ciccone et al., 2004b). However, the possible involvement of GnIH in the rearing of chicks after hatch has not been examined in a precocial species.
Seasonal changes in the expression of RFRP in mammals resemble those seen for GnIH in birds. In sheep, in which reproduction is activated by short

days and inhibited by long days, during the breeding season there was less RFRP expression and immunoreactivity in the hypothalamus, as well as fewer RFRP fibres connecting to GnRH neurones, compared to the non-breeding season (Smith et al., 2008). Similarly, in the female pig, expression of GnIH and its receptor in the hypothalamus were lowest during estrous and highest in proestrus and diestrus (Li et al., 2012).

5.1.2.2 Context-dependent changes

There is evidence that, in European starlings, GnIH is affected by social and breeding status, as birds who were more successful breeders were shown to have less GnIH in their brains (Calisi et al., 2011). Once again in starlings, GnIH levels in the brain were highest on the first day of incubation and on the first day after hatch, while in rats they decreased directly after birth, demonstrating that this hormone's activity patterns differ in different species and are likely to be influenced by an animal's life history (Calisi et al., 2016). In both starlings and rats GnIH also increased after loss of young (Calisi et al., 2016). This increase is somewhat surprising since resuming sexual activities (and thus a decrease in GnIH) in order to replace the lost progeny seems more logical but as GnIH is also increased by stress, this response may exist in order to inhibit reproduction in conditions where there is potential danger present, e.g. a predator. In any case, this finding shows that the GnIH system responds quickly to changes in the environment. This plasticity may be of great importance in opportunistically breeding species. In agreement with this hypothesis, in Rufous-winged sparrows living in the Sonoran desert, which breed after the irregular monsoon season, birds caught during the monsoon season had fewer GnIH-immunoreactive neurones in their brains than birds caught before this season (Small et al., 2008). In the same study, GnRH neurones were found to be larger during the monsoon season (Small

et al., 2008).

In addition to seasonal changes, the reproductive stage, as well as the sex of the animal can also influence the effects of GnIH. It was found in the chicken that activation of the GnIH receptor inhibited signalling from the GnRH type 3 receptor, thus inhibiting the secretion of gonadotropin and the ratio of GnIH vs GnRH receptors in the pituitary gland changed with sexual maturation in this species in favour of GnRH receptors so that pituitary sensitivity shifted from inhibitory to stimulatory, unlocking the release of gonadotropin (Shimizu and Bédécarrats, 2010). The effects on GnIH of LH and FSH were also dependent on the reproductive stage in goldfish (Moussavi et al., 2013, Moussavi et al., 2012). In the striped hamster, breeding males had higher RFRP expression in the hypothalamus than non-breeding (both juvenile and aged) males, while the opposite was true for females when breeding females were compared to aged females (Zhao et al., 2014).

5.1.3 Effects of gonadotropin inhibitory hormone on feeding

Aside from its role in reproduction, GnIH has also been shown to stimulate feeding in domestic chicks (Tachibana et al., 2005), rat (Johnson et al., 2007), and Pekin duck (Fraley et al., 2013). Central infusion of GnIH also increased feeding in sheep, mice and cynomolgus monkeys (Clarke et al., 2012). In female Syrian hamsters, food restriction increased the number of GnIH-immunoreactive neurones co-expressing c-fos, and this activation of GnIH neurones was positively correlated with food hoarding (Klingerman et al., 2011). It is possible that GnIH acts as an innate hunger factor, as it has been demonstrated to stimulate feeding similarly in high and low body weight chicken lines, and its expression was found to be increased by fasting (McConn et al., 2016). In agreement with these results, food deprivation for 48 hours in the Pekin duck induced c-fos expression in approximately 50% of

GnIH-immunoreactive neurones in the brain (Fraley et al., 2013). By contrast, in chicks only the LHy showed an increase in c-fos expression after feeding stimulated by central injections of GnIH, suggesting that GnIH may induce feeding through effects on this particular brain area (Calisi et al., 2016). The orexigenic neuropeptide Y (NPY) and anorexigenic pro-opiomelanocortin (POMC) may be involved in this process, as in the above study, the expression of the former was increased and the latter decreased in the hypothalamus as a whole (Calisi et al., 2016). However, when only samples containing the LHy were analysed, these two peptides remained unchanged but the expression of the orexigenic melanin-concentrating hormone (MCH) was increased (Calisi et al., 2016), also suggesting a possible interaction between this peptide and GnIH. Another study in chicks proposed that the orexigenic effect of GnIH is mediated by central opioid receptors as GnIH-stimulated feeding behaviour after central injection of the peptide was blocked by co-injection of an opioid receptor antagonist (Tachibana et al., 2008). Interestingly, while both GnIH and NPY appear to stimulate feeding, GnIH negatively regulated NPY in mouse arcuate nucleus (Jacobi et al., 2013). Also notable is the finding that while GnIH administration increased c-fos expression only in orexigenic neurones (NPY neurones) in rats, it increased expression in both orexigenic and anorexigenic neurones (both NPY and POMC neurones) in sheep (Clarke et al., 2012). In Abert's towhee - a seasonally breeding songbird species in which food availability influences reproduction - GnIH and NPY seemed to be responsible for integrating information on energetic status, as the number of NPY-immunoreactive neurones increased while the size of GnIH-immunoreactive neurones decreased when captive adult males underwent food restriction for 2 or more weeks, while food restriction also decreased plasma LH and testosterone, as well as the size of the cloacal protuberance, which is a secondary sexual characteristic dependent on testosterone (Davies et al., 2015). The dual involvement of GnIH in both breeding and food intake as demonstrated in the sheep and rat (Clarke et al., 2012), as well as the the Pekin duck (Fraley et

al., 2013) and the towhee (Davies et al., 2015) suggests that GnIH is a molecular switch between reproduction and feeding.

A connection between feeding and incubation exists in the chicken as incubating hens have a decreased food intake in exchange for spending more than 95% of their time sitting on the nest (Zadworny et al., 1988). However, it is not known whether this incubation-related decrease in feeding is connected to GnIH in the brain.

5.1.4 Aim and hypothesis

The aim of this study was to characterise the changes in GnIH expression and synthesis throughout the reproductive cycle of the domestic hen and test the hypothesis that GnIH may be involved in the control of incubation and the care of chicks after hatch. The possibility that GnIH neurons in the LHy may be involved in the decrease in feeding in incubating hens, observed in previous studies, was also examined. For this purpose, adult hens were allowed to go through their natural reproductive cycles and brains were collected at different time points to provide a detailed picture of the changes in GnIH levels in the brain. This is, to the author's knowledge, the first study to examine GnIH in the chicken brain at two different time points during incubation and in early rearing.

Hypothesis:

Based on the available literature in birds cited above, it was hypothesised that GnIH mRNA expression and immunoreactivity would increase in the PVN of the domestic hen with the onset of incubation and remain high throughout this period, possibly in relation to the suppression of sexual

activity in maternal birds, while GnIH mRNA expression and immunoreactivity in the LHy would decrease at this time and remain low throughout incubation, possibly in relation to a decrease in feeding during this period observed in previous studies.

5.2 Materials and methods

5.2.1 Animals and housing

The same birds from the F1 generation of a cross between Silkie and White Leghorn as in Chapter 4 were used in these experiments. More information on animals and housing, as well as the experimental design, is provided in that chapter. Briefly, adult female chickens were allowed to go through their natural reproductive cycle and brains were collected at the following time points: Laying (continuously laying eggs), Onset (day 3 of sitting on the eggs), Incubating (day 14 of sitting on the eggs) and Rearing (the first day after hatch).

5.2.2 Tissue collection

Brains were collected and the reproductive status of the birds was confirmed as described in Chapter 4 of this thesis.

5.2.3 Immunohistochemistry

An introduction to this procedure can be found in Chapter 2. The Vectastain Elite Rabbit IgG ABC Kit was used to visualise GnIH in the brain (see Chapter 3).

Sections covering the caudal part of the PVN after the anterior commissure were included in the procedure. Damaged sections were disregarded in subsequent analysis. Between 4 and 6 sections were analysed for each bird on the left and right side of the PVN and the average of those measurements was used in the final analysis.

This immunohistology protocol was developed in the Brunton Lab based at The Roslin Institute. After experimenting with different fixatives, it was concluded that ice-cold acetone produced optimal results when working with thin fresh frozen sections. Therefore, this fixative was used in the procedure below. Acetone was cooled on ice, slides were fixed in acetone in fume hood for 10min and then left to dry in the fume hood for 30min. Slides were washed three times for 5 min in 1xPBS and then incubated for 1hour in blocking solution consisting of 2% NGS in 1xPBS. GnIH primary antibody was diluted in blocking solution (same as above) to a concentration of 1:1000. A hydrophobic pen was used to draw around the tissue and 500µl antibody were applied per slide. Slides were left to incubate in humidifying racks for 48 hours.

After this incubation slides were washed three times for five minutes in 1xPBS on a shaking platform at 120 rpm and then given two more 15-min washes off the shaker in 1xPBS. The use of the shaking platform aided the effective washing of the slides. Endogenous peroxidases were quenched for 15 min in a solution containing 20% methanol, 50% 2xPBS, 5% H_2O_2. Slides were then washed in 1xPBS for 15 min. Biotinylated anti-rabbit antibody was diluted in 1xPBS with 15% NGS to a concentration of 1:100. 500µl were

applied per slide and slides were incubated in humidifying racks for 60 min before being washed three times in 1xPBS for 5 min. ABC solution was prepared 30 min before use and consisted of 1.5µl/ml reagent A and 1.5µl/ml in 1xPBS. Once again 500µl per slide were applied and slides were incubated in a humidifying rack 60 min before being washed three times in 1xPBS for 5 min. 1% DAB solution was prepared as above and 0.03% H_2O_2 were added just before use and mixed well by inverting the bottle vigorously. Slides were incubated in DAB solution for 10 min and then washed five times for 5 min in 1xPBS. Slides were then dehydrated through an alcohol series and coverslipped as above.

5.2.4 DIG *in situ* hybridisation

An introduction to this procedure can be found in Chapter 2.

The probe template used in this procedure was designed and provided by Dr Yasuko Tobari and had the following sequence:

>chicken GnIH plasmid sequence, 861 bases.

GGAAGTCATTTCAACCCAGAAGTTTATTCTGCTTACTTTGGCTACAGTGG
CGTTTCTAACACCGCATGGTATGTGCCTAGATGAACTAATGAAATCCAG
CCTGGAGAGCAGAGAAGAAGATGATGATAAATATTACGAGATTAAAGAC
AGTATCTTGGAGGAAAAGCAGAGGAGTCTGAATTTTGAAGAAATGAAAG
ACTGGGGATCAAAAAATTTCCTTAAAGTGAACACCCCTACAGTAAACAAA
GTGCCAAATTCAGTTGCTAATTTACCTCTTAGGTTTGGAAGAAGCAATCC
AGAAGAAAGAAGCATTAGGCCGAGTGCTTATTTGCCTTTGAGATTTGGA
AGAGCTTTTGGAGAGAGCCTATCTAGGCGTGCTCCAAATTTATCGTACA
GGCTTGGGAGATCTCCACTTGCTAGAAGTTCTATTCAATCCCTTCTGAAT

CTGCCACAGAGATTCGGGAAGTCAGTGCCCATCAATCTATCTCAAGGTG
TCCAGGAGTCTGAACCAGGGATGTGAATTCTAACAATCACATTCAAGTT
ATCAAGTTATGAAGATGGAAACTGAAAGACTTGGAAAAGATGCAGCGTG
TATTCAAAACTAAGGATCTTGAGAAGGGAATGTATATGAGAACTGAAATG
CAAACCTGCATTACGTTATTATTATGTTGTAATGTGTGTATTCTGTACAAA
CAATTGATGTTTAAGGCATTGATAACTGTAGTGAGCATTGTACCAGTTTA
CTTCACTGGTGGCGTAGTACAAATTCTATTTACTTCTGTGTGACTTCTGT
GTGACTGATACTCTGTTGGCTGTAAAAATTAATTTAAAAACAATAGCTGT
GGAATTGAAAGAATTTTG

5.2.4.1 Preparation of culture medium and ampicillin plates

In this step, bacterial cells were transfected with the riboprobe containing plasmid and grown in culture.

1.75g LB-Agar and 50ml ddH_2O were used per plate. The agar was dissolved in the ddH_2O, autoclaved and cooled down slowly to 50°C. 50mg ampicillin powder (Sigma) was dissolved per 1ml sterile ddH_2O and stored at 4°C until needed. 0.1ml of this was added to every 50ml agar (once it had cooled to 50°C) to a final concentration of 100µg/ml. Solution was immediately poured into plates in the fume hood and agar was left to set. Plates were kept at 4°C until use. Autoclaved LB-broth was used as culture medium. DH5-α competent cells which were kept at -70°C were thawed slowly on ice and 100µl were added to each incubation tube. The plasmid solution was also thawed on ice and then 1µl was added to each incubation tube. Incubation tubes were kept on ice for 1-2 hours to allow plasmid to precipitate onto the outside of the cells. Cells were then heat-shocked for 75 sec in a water bath at 42°C to promote movement of DNA into the competent cells after which the tubes were cooled down to room temperature on ice.

0.4ml LB-broth was added to the tubes in a fume hood and tubes were placed on a shaking incubator for 30 min at 37°C. Meanwhile ampicillin plates were removed from freezer and allowed to dry for several minutes in a fume hood. 200µl medium containing the transformed cells were spread over the ampicillin plates and they were stacked upside down and placed in a 37°C incubator overnight.

The next day, plates were removed from incubator and stored at 4°C until use. In a fume hood, a single colony was picked per plate and transferred into 1.5ml LB-broth in an incubation tube. Tubes were incubated on a shaking incubator for 4-5 hours, after which tube contents were transferred to conical flasks containing 50ml culture medium and grown on a shaking incubator overnight.

5.2.4.2 Plasmid DNA purification

Plasmid DNA was purified from the cultured cells using Qiagen Plasmid Midi Prep Kit (Cat. # 12145) following the manufacturer's protocol. The protocol can be found in the QIAGEN Plasmid Purification Handbook which can be downloaded here: https://www.qiagen.com/us/resources/resourcedetail?id=46205595-0440-459e-9d93-50eb02e5707e&lang=en

The yield was determined by Nanodrop. As the yield was sufficiently high, no agarose gel analysis of the efficiency of the procedure was necessary. Plasmid DNA was stored at -20°C.

5.2.4.3 Plasmid linearization

It is necessary for plasmid DNA to be linearised in order to serve as a template. This is achieved through enzyme restriction digestion at specific restriction sites on the plasmid. In our case the plasmids were designed to contain restriction sites for the Xba1 and Spe1 enzymes.

Water bath was heated to 37°C and the required reagents (plasmid DNA, restriction enzymes and their relevant buffers, BSA) were thawed on ice. The components of the linearisation reaction mix were added to a sterile Eppendorf. The antisense (probe) mix contained 20% plasmid DNA, 4% Xba1 restriction enzyme, 10% Buffer D (corresponding buffer to the Xba1 enzyme), 10% bovine serum albumin (BSA) and 56% DEPC-treated ddH_2O. The sense mix was the same except it contained the Spe1 enzyme and its corresponding buffer (Buffer S) instead of Xba1. A double-digest was also prepared which contained both enzymes and a multipurpose buffer (Multicore buffer M). The tubes were vortexed and centrifuged briefly at top speed at RT and then incubated in water bath at 37°C for 2 hours.

5.2.4.4 Phenol/chloroform extraction of protein from the nucleic acid preparation

This step was performed in order to clear the linearised plasmid of any proteins. A matching volume of Phenol:Chloroform Isoamyl Alcohol (stored at 4°C) was added to the digested plasmid and vortexed. The tubes were them centrifuged at top speed for 5 min. The top layer which contained the DNA was carefully removed to a new Eppendorf.

An equal volume of Chloroform:Isoamyl Alcohol was added. The tubes were again vortexed and centrifuged as above. The top layer was once again removed to a fresh eppendorf. 5M NaCl equal to 1/10 of the volume of the DNA along with 100% ethanol equal to 2x the total volume of DNA and 5M NaCl were added to the tube and the contents were mixed by finger flicking and left on dry ice for 10 minutes. After the first 5 min contents were thawed by warming in hand and then replaced on the dry ice for the remaining 5 min. Tubes were centrifuged at 13,000rpm for 15 min at RT. The supernatant was carefully decanted without disturbing the very small pellet and the pellet was left in the fume hood until dry before being rehydrated in DEPC-treated H_2O equal to about $1/6^{th}$ of the initial volume of the linearised plasmid. This now ready to use template was stored at -20°C until use.

5.2.4.5 Gel Electrophoresis of linearised fragments

This step was performed in order to confirm linearisation of the plasmid and presence of fragments of the correct size. TBE buffer (Tris-borate-EDTA buffer) was diluted to 1x working concentration in ddH_2O. 1% agarose gel was prepared by adding 1g agarose for every 100ml TBE buffer, heating in microwave until agarose was completely dissolved and making up the evaporated volume with ddH_2O. 10µl SYBR Safe was added per 100µl and the gel was left to set with a comb in a gel mould. Gel was loaded in a gel electrophoresis tank in 1xTBE buffer. 2µl template and 1µl loading buffer were added to 7µl DEPC-treated H_2O for the sense and anti-sense probes. 5µl double digest template and 1µl loading buffer were added to 4µl DEPC-treated H_2O. 5µl of each of these solutions were loaded onto the gel along with 5µl 100bp DNA ladder. The gel was run at 120V for about 45 min. Gel was imaged under UV light to confirm the presence of bands of the correct size.

5.2.4.6 DIG labelling of RNA Probes

DIG instead of radioactive ISH was used in this case because this procedure is safer and much quicker than radioactive ISH, as well as cheaper. In addition, the procedure had already been shown to work on quail in the lab following this protocol by Dr Yasuko Tobari. Since by the time these procedures were performed, time constraints were being faced, and since the results of the initial tests with DIG ISH were considered satisfactory, it was determined that this method was a better choice under the circumstances.

In this procedure the DNA template was used to create a DIG-labelled riboprobe. SP6/T7 Trascription Kit and DIG RNA Labeling Mix were used for the purpose. SP6 and T7 are RNA polymerases which recognise specific sites present respectively on the antisense and sense templates used here. Two water baths were heated to 37°C and 40°C respectively while reagents were thawed on ice. The labelling mix (20µl in this case) for the antisense template (probe) consisted of 20% antisense template, 45% sterile ddH_2O, 10% 10x NTP labelling mix, 10% 10x transcription buffer, 5% RNase inhibitor and 10% SP6 RNA polymerase. The mix for the sense template was the same except SP6 RNA polymerase was replaced with T7. A control mix was also prepared using one of the templates (we used the antisense template but either one could have been used). This mix consisted of 10% purified template 55% ddH_2O, 10% 10x DNP labelling mix, 10% 10x transcription buffer, 5% RNase inhibitor and 10% the corresponding polymerase. The labelling mixes were incubated in a water bath at 37°C for T7 and 40°C for SP6 for 2 hours. After the first hour tubes were removed briefly and mixed by flicking before being returned to the water baths. After this incubation, 1µl of DNase1 enzyme per 10µl was added to each tube to remove the DNA template. Tubes were mixed well with a pipette tip and placed in a 37°C

water bath for another 15min. The reaction was terminated by adding 2µl 0.2M EDTA (pH 8.0) and mixing with a pipette tip.

5.2.4.7 Purification of DIG labelled RNA

In order to precipitate the labelled RNA transcript, 76µl ddH_2O, 10µl 3M sodium acetate and 250µl 100% ethanol were added to each 20µl reaction mix, mixed well and incubated in a water bath at 70°C for 30 min. Tubes were centrifuged at 13,000rpm for 10 min at 4°C. Supernatant was discarded and the pellet was washed with 500µl ice-cold 70% ethanol. Tubes were centrifuged again at top speed for 5 min at 4°C and alcohol was carefully removed without dislodging the pellet. Pellets were air-dried in fume hood for approx 1 hour and resuspended in 50µl DEPC-treated H_2O. Labelled probes were stored at -20°C.

5.2.4.8 Immunological detection of labelled probe with a charged membrane

DIG Wash and Block Buffer Set was used in this procedure according to the manufacturer's protocol. 10x stock buffer solutions were all diluted to 1x with sterile ddH_2O and the detection buffer's pH was adjusted to 9.5. 10x blocking solution was diluted to 1x in 1x maleic acid buffer. The control DIG-labelled DNA was serially diluted to 5ng/µl, 1ng/µl, 10pg/µl, 3pg/µl, 1pg/µl, 0.3pg/µl, 0.1pg/µl, 0.03pg/µl and 0.01pg/µl. The same dilution series was prepared for the sense and anti-sense probes as well. 1µl of each concentration was applied to a strip of positively charged membrane. The nucleic acid was fixed to the membrane by cross-linking under UV light (0.240 Joules/cm). After

fixation, the membrane was transferred to a Petri dish and washed for 5 min in 1x wash buffer. Membrane was then incubated for 30 min in 1x blocking solution.

Meanwhile anti-DIG-AP IgG antibody was centrifuged at 6 000 x g for 5 min and diluted to a concentration of 1:5000. Blocking solution was drained from the membrane and it was submerged in antibody solution and incubated at RT for 30min before being washed twice for 10 min in 1x washing buffer. The membrane was then equilibrated by immersing in 1x detection buffer for 5min. 200µl NBT-BCIP were added to each 10ml detection buffer before submerging the membrane in this colour substrate solution protected from light for 2-3 hours. The colour reaction was halted by rinsing in ddH_2O twice for 5 min. The membrane was visually assessed to confirm the presence of labelled probes.

5.2.4.9 Prehybridisation

Stock 10xPBS solution (1.4M NaCl, 0.027M KCl, 0.1M Na_2HPO_4, 0.018M KH_2PO_4) was prepared with 1ml/l DEPC and shaken well. pH was adjusted to 7.4 and the solution was autoclaved. DEPC-treated H_2O was used to dilute this stock to 1xPBS. 4% PFA was made up in DEPC-treated 1xPBS.

0.1M TEA was used with 250µl acetic anhydride per 100ml in the acetylation step to prevent non-specific binding of the negatively charged probe to positively charged tissue.

Boxes containing pre-selected slides were removed from -70°C freezer and allowed to reach RT for 2 hours before being processed in sealed boxes with silica gel bags. Slides were then fixed in 4% ice-cold PFA in DEPC-treated 1xPBS for 10min. Slides were washed in DEPC-treated 1xPBS three times for five minutes and then incubated in TEA/AA solution where AA was added

and stirred just before use. Following this, slides were incubated in for 30min in 1% triton in DEPC-treated 1xPBS and washed again in DEPC-treated 1xPBS three times for 5 min.

Slides were placed in humidifying chambers with DEPC-treated H_2O. Stock 2x riboprobe hybridisation buffer (24% 5M NaCl (292.2g/l), 2% 1M Tris, pH 7.6 (149g/l), 4% Denhardt's solution (50x), 0.8% 500mM EDTA, 2% salmon testes single-stranded DNA (10mg/ml), 50% Dextran sulphate (0.4g/ml), 0.5% Yeast tRNA and 16.7% DEPC-treated H_2O) was diluted with an equal volume of deionised formamide to make 1x hybridisation buffer and 500µl were applied per slide. Slides were incubated for 2 hours.

5.2.4.10 Hybridisation and post-hybridisation washes

To make the hybridisation solution, 1µl of DIG-labelled probe was added to every 1ml of 1x hybridisation buffer, vortexed and incubated in a water bath at 80°C for 5 min. Reaction was stopped on ice. Hybridisation buffer was drained from slides and 200µl hybridisation solution was applied per slide. Pieces of parafilm were used to cover the slides and spread the solution. Slides were arranged on glass plates in plastic humidifying boxes with DEPC-treated H_2O. Boxes were sealed and slides were incubated for 16 hours at 72°C in a hybridisation oven.

After hybridisation each slide was swiped through a jar full of 5xSSC at 72°C to remove parafilm. Slides were arranged in racks and washed four times for 30 min in 0.2xSSC at 72°C. Slides were then washed one more time for 5min in 0.2xSSC at RT.

Buffers for the following steps were diluted as described above.

Slides were washed in 1x wash buffer for 5min and incubated in blocking solution for 60 min at RT. Anti-DIG-AP IgG was prepared as above. 500µl of the antibody were applied per slide and slides were left to incubate in humidifying rack overnight. Solution was drained and slides were washed three times for 5 min in 1x wash buffer. Slides were returned to humidifying rack and tissue was equilibrated with 500µl 1x detection buffer per slide for 15min. Colour solution was prepared as above and 400µl were applied per slide after draining detection buffer Tissue was incubated in colour substrate solution for 5 hours protected from light at room temperature. Slides were placed in racks and washed twice for 5 min in 1xPBS, after which they were rinsed for 5 min in ddH_2O.

5.2.4.11 Counterstaining

To counterstain the slides, 500µl Nuclear Fast Red was applied to each slide and slides were left to incubate for 20 min. Slides were rinsed under running tap water for 10 min and then once again in ddH_2O for 5 min. Sections were sealed with aqueous based mounting medium (CC Mount).

The sense probe was used as a negative control. Figure 5.1 shows sections hybridised with the sense and antisense probes.

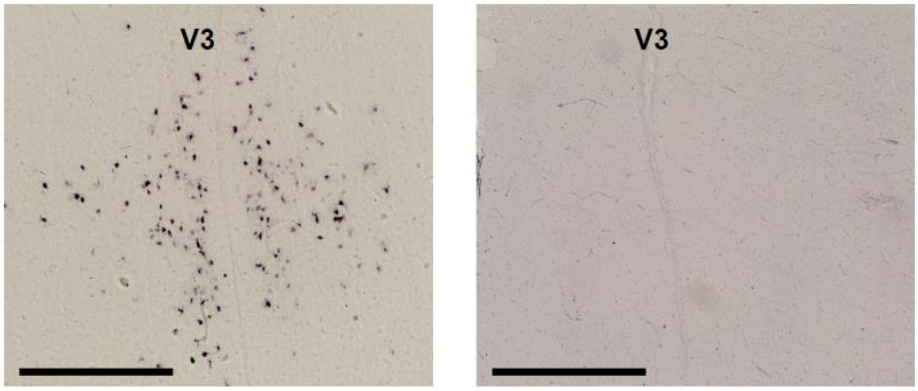

Figure 5.1 DIG ISH in the chicken PVN with antisense and sense probes.
GnIH mRNA signal (dark staining) in chicken brain sections (region shown is the PVN) hybridised with antisense (left) and sense (negative control, right) probe. Scale bar = 500µm.

5.2.5 Statistical analysis

Results were analysed using One-way ANOVA and Tukey's post-hoc test or, where the data were not normally distributed, Kruskal-Wallis ANOVA on ranks and Dunn's post-hoc test were performed. Details can be found in Chapter 2.

5.3 Results

5.3.1 Body weight of the hens

The average weight at cull for each of the four groups of birds is shown on Figure 5.2.

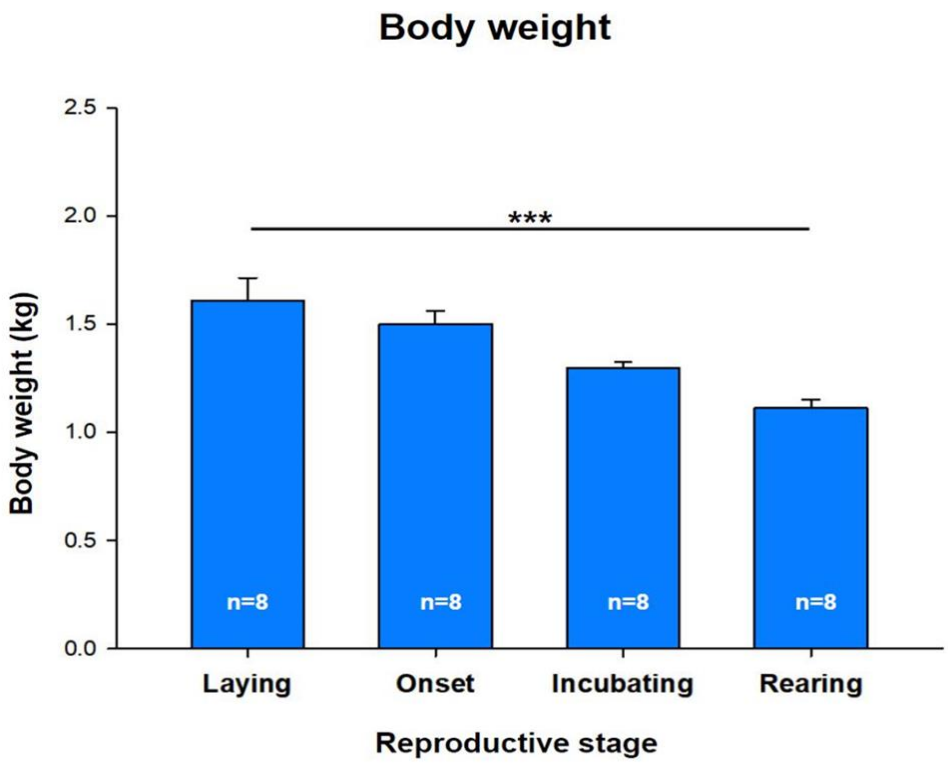

Figure 5.2 Body weight of hens throughout the reproductive cycle.

There was a significant difference between groups in the body weight of Laying, Onset, Incubating and Rearing hens (Kruskal-Wallis ANOVA on ranks, $H_{(3)}$=18.423, p<0.001). There was a significant difference between the weight of Laying birds and birds on the first day of rearing (Dunn's test, p<0.001). Data are presented as mean + SEM (n=8 per group). *** denotes p<0.001.

5.3.2 Distribution of gonadotropin inhibitory hormone mRNA-expressing and gonadotropin inhibitory hormone-immunoreactive neurons in the hypothalamus of hens

Groups of GnIH mRNA-expressing neurons were observed in the PVN and LHy of hens (Figure 5.3). This distribution was in agreement with previous studies. The distribution of GnIH-immunoreactive neurons matched that of GnIH mRNA-expressing cells, although on many of the sections the LHy was damaged by the IHC procedure and GnIH-immunoreactive neurons there were impossible to quantify.

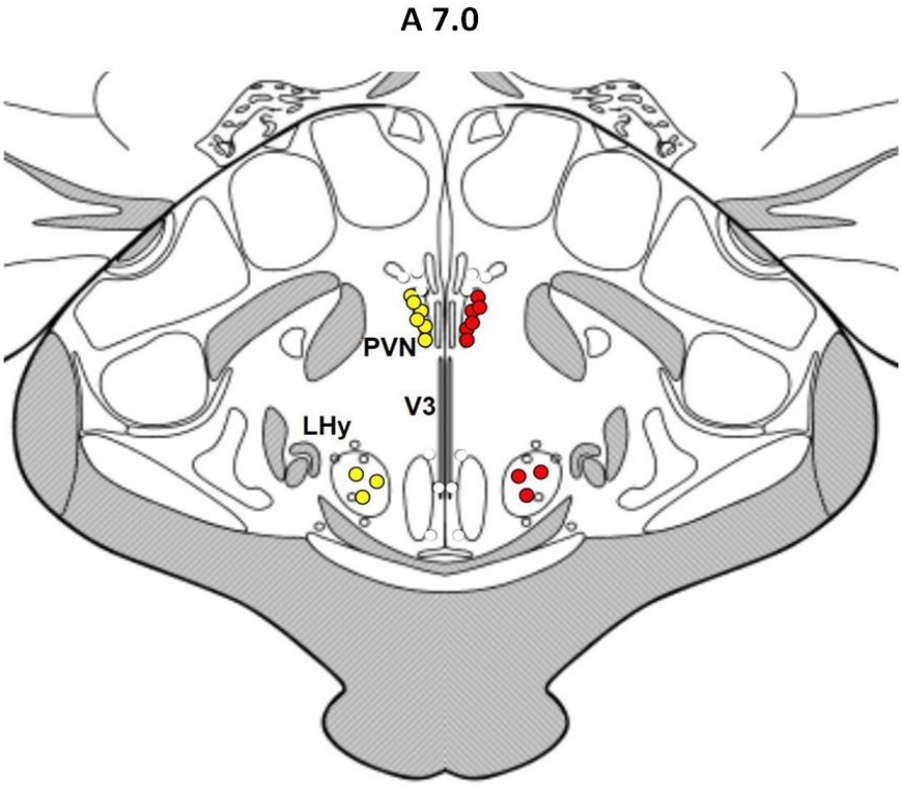

Figure 5.3 Distribution of GnIH mRNA expression and GnIH immunoreactivity in the hypothalamus of the domestic hen. Schematic diagram of a coronal section illustrating the distribution of GnIH mRNA-expressing neurons (yellow dots) which matches that of GnIH-immunoreactive neurons (red dots) in the brain of female Silkie x White Leghorn cross chicken. Illustrations are redrawn, with the given coordinates, from the stereotaxic atlas of the chick brain (Kuenzel and Masson, 1988) where 'A' followed by a number signifies a brain plate the given number of millimetres anterior to a zero reference plane. LHy, Regio lateralis hypothalami (lateral hypothalamic area); PVN, nucleus paraventricularis magnocellularis; V3, ventriculus tertius (third ventricle).

5.3.2 Gonadotropin inhibitory hormone mRNA-expressing cells in the paraventricular nucleus and the lateral hypothalamus of hens throughout the reproductive cycle

Photomicrographs of neurones positive for GnIH mRNA in the PVN and LHy throughout the reproductive cycle, along with graphical representation of the results of the statistical analysisare shown on Figure 5.4 and Figure 5.5 respectively. No statistically significant difference between groups was found in either the PVN (One-way ANOVA, $F_{(3,25)}$=0.918, p=0.446) or the LHy (Kruskal-Wallis ANOVA, $H_{(3)}$ = 3.971 p = 0.265).

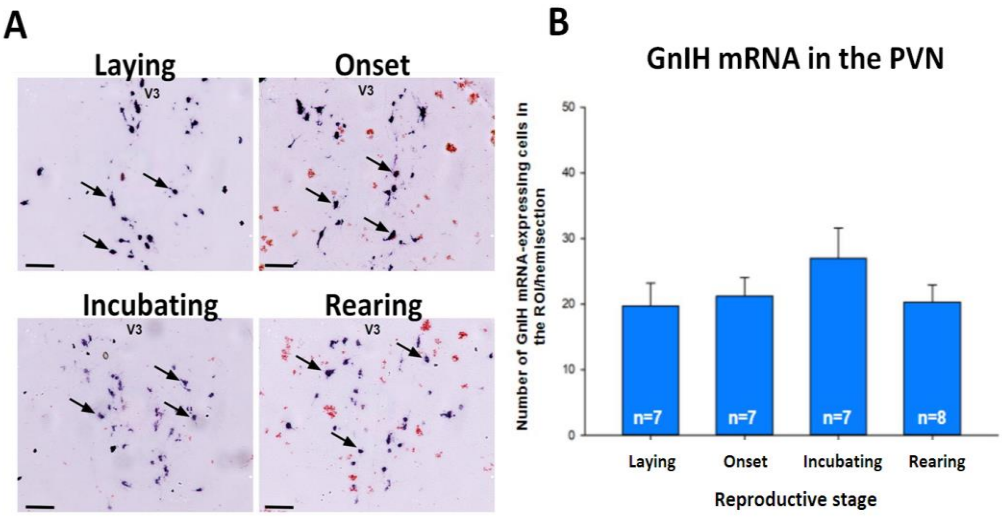

Figure 5.4 GnIH mRNA-expressing cells in the PVN of hens throughout the reproductive cycle.
A. DIG *in situ* hybridisation staining for GnIH mRNA in the PVN of Laying, Onset, Incubating and Rearing hens. Arrows denote labelled cells (stained in purple). Red spots are the result of staining with Nuclear Fast Red. Although difficult to see, the third ventricle is present on all images and its placement is indicated (V3). Scale bars = 100µm.
B. No statistically significant difference between groups was found in the number of neurons showing GnIH mRNA expression in the PVN (One-way ANOVA, $F_{(3,25)}$=0.918, p=0.446). n=7-8 per group. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to mistakes during the procedure, such as the tissue drying out, leading to loss of certain slides.) Data are presented as mean + SEM.

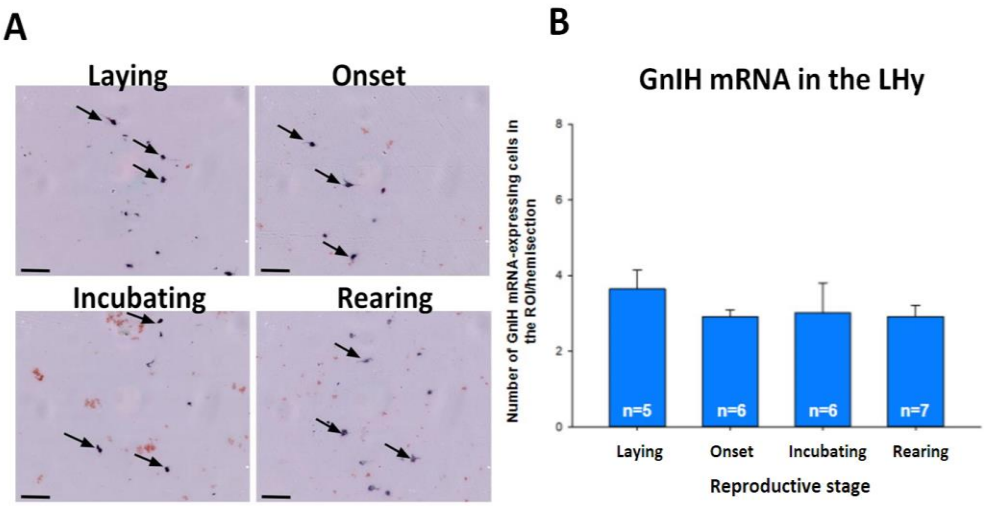

Figure 5.5 GnIH mRNA-expressing cells in the LHy of hens throughout the reproductive cycle.

A. DIG *in situ* hybridisation staining for GnIH mRNA in the LHy of Laying, Onset, Incubating and Rearing hens. Arrows denote labelled cells (stained in purple). Red spots are the result of staining with Nuclear Fast Red. Scale bars = 100µm.

B. No statistically significant difference between groups was found in the number of neurons showing GnIH mRNA expression in the LHy of hens (Kruskal-Wallis ANOVA, $H_{(3)}$ =3.971, p= 0.265). n= 5-7 per group. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to mistakes during the procedure, such as the tissue drying out, leading to loss of certain slides, as well as this region being damaged on some slides.) Data are presented as mean + SEM.

5.3.3 Gonadotropin inhibitory hormone-immunoreactive cells in the paraventricular nucleus throughout the reproductive cycle

Photomicrographs of GnIH-immunoreactive cells in the PVN of hens in the four groups along with graphical representation of results are shown on Figure 5.6.

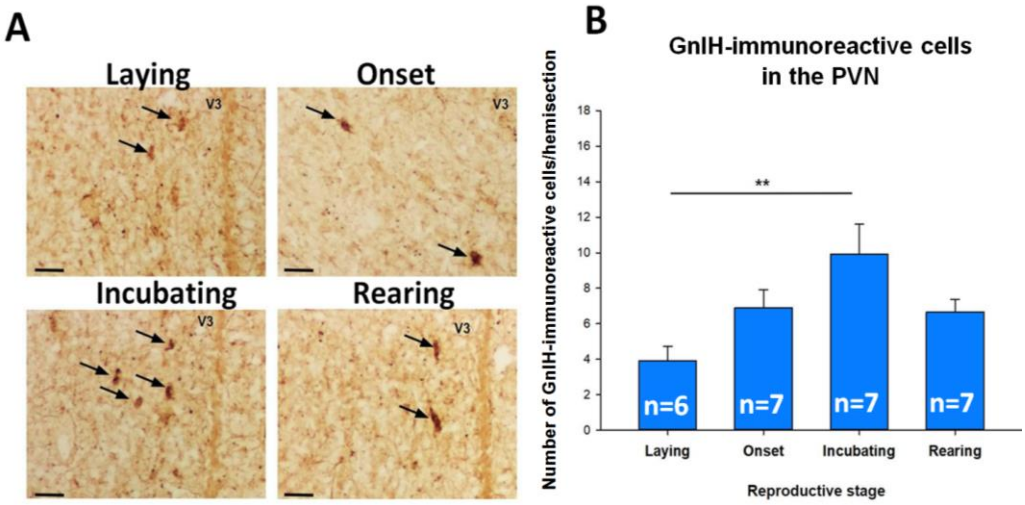

Figure 5.6 GnIH-immunoreactive cells in the PVN of hens throughout the reproductive cycle.
A. Photomicrographs of immunohistochemical staining for GnIH in the PVN of Laying, Onset, Incubating and Rearing hens. Arrows denote labelled cells. The position of third ventricle is indicated (V3). Scale bars = 200µm
B. There was a significant difference between groups (One-way ANOVA, $F_{(3,23)}$=4.395, p=0.014) in the number of GnIH-immunoreactive neurones in the PVN of hens. Post-hoc comparisons (Tukey's test) revealed that hens on day 14 of incubation (Incubating) had a significantly greater number of GnIH-immunoreactive cells than Laying in this brain area (p=0.008). n= 6-7 per group. (8 birds per group were included in the initial procedure but some individuals were excluded from analysis due to the region of interest being damaged on some sections, most likely during the IHC washes.) Data are presented as mean + SEM. ** denotes p<0.01.

There was a significant difference between groups in the number of GnIH-immunoreactive cells in the PVN (One-way ANOVA, $F_{(3.23)}$=4.395, p=0.014).

Post-hoc comparisons (Tukey's test) revealed that Birds on day 14 of incubation had significantly more immunoreactive cells than laying birds ($p=0.008$). Damage to sections (likely during washes due to the thinness of the tissue and the fact that this region is closer to the edge of the section than the PVN and gets more easily eroded) made it impossible to analyse the number of GnIH-immunoreactive neurons in the LHy of hens.

5.4 Discussion

5.4.1 Gonadotropin inhibitory hormone and incubation

The results presented in this chapter demonstrate that GnIH immunoreactive levels in the hypothalamus of the chicken change throughout the reproductive cycle, with incubating birds having a higher number of GnIH-immunoreactive neurones in their PVN than birds laying eggs. These results are in agreement with previous findings in the chicken (Ciccone et al., 2004b), and also generally correspond to observations in other birds and mammals which show that GnIH is high at times when the reproductive system is inhibited, regardless of whether this inhibition is related to unpredictable factors like stress or seasonal changes in reproduction (Amorin and Calisi, 2015; Bentley et al., 2003; Calisi et al., 2008; Ciccone et al., 2004a; Dixit et al., 2017; Li et al., 2012; Maddineni et al., 2008; McGuire et al., 2011; Moussavi et al., 2013; Osugi et al., 2004; Satake et al., 2001; Small et al., 2008; Smith et al., 2008; Zhao et al., 2014). However, in this study, a significant difference between the incubating and laying group was only observed in the number of GnIH-immunoreactive neurons in the PVN, and not in the number of mRNA-expressing cells. This could be due to differences in the sensitivities of the two procedures or differences between

the rates of transcription and translation. As data presented in Chapter 4 of this thesis demonstrates, the increase in GnIH during incubation coincides with a period when testosterone was undetectable in plasma by ELISA assay. This supports the hypothesis that the function of GnIH at this stage of the cycle is to downregulate the expression of sex steroids and inhibit reproduction. However further studies are necessary to determine how crucial the action of this peptide is for the onset and maintenance of incubation. This could be achieved through studies using GnIH antagonists, gene silencing or knockout and observing the effect of these treatments on the onset and maintenance of incubation. The timing of GnIH increase observed does not match the timing of increased GnIH in another avian species - the European starling. GnIH was highest in starlings on the first day of incubation and the first day after hatch (Calisi et al., 2016), while in the chicken, it peaked much later in incubation and decreased to levels similar to those of laying birds by the first day of rearing. There is a difference in these two species in the duration of incubation and mode of care for the young. Incubation in the starling lasted 11-12 days (Calisi et al., 2016), while in the chicken it lasted 21 days. This may explain why the timing of the peak in GnIH was shifted later in chickens. However, the lack of a significant increase early on suggests that GnIH may not be necessary for the onset of incubation in hens and a different mechanism may be responsible for the decrease in sex steroids which is observed even in early incubation. Administration of GnIH antagonists in incubating hens and subsequent observation of any changes in incubating behaviour and any signs of sexual behaviour could provide more data on its importance at this stage of the reproductive cycle.

5.4.2 Gonadotropin inhibitory hormone and the care of chicks post-hatch

As mentioned above, in starlings, there was a second peak in GnIH on the first day after hatch (Calisi et al., 2016). The explanation for the lack of this second increase in the chicken may lie in this bird's precocial nature.

Starlings are altricial species and parents are crucial to the survival of hatchlings whereas newly hatched precocial chicks can feed independently and possess a certain level of autonomy from the mother. The altricial nature of starlings may necessitate the presence of GnIH after hatch to suppress sexual behaviours in favour of parental behaviours which may not be of the same importance in the chicken. A study involving GnIH antagonists in hens rearing chicks may help determine whether this peptide is of any importance for maternal care after hatch.

5.4.3 Gonadotropin inhibitory hormone and feeding during incubation

Chickens have been shown to decrease their food intake by more than 60% during incubation (Zadworny et al., 1988). In agreement with this, the weights of the birds in this experiment showed a steady decrease throughout incubation, with rearing hens having significantly lower average weights at cull compared to layers (Figure 5.2). Therefore, it is interesting to note that while GnIH is an orexigenic factor in chicks (Tachibana et al., 2005), its increase in the PVN in this case corresponds to a time when the hen feeds very little. Fasting has been shown to increase GnIH (McConn et al., 2016) but this was demonstrated in juveniles at 56 days of age which were denied food, whereas the present study involved adult incubating hens which had access to food *ad libitum,* but their feeding was internally suppressed. It has

been suggested that GnIH may control feeding in chicks through the LHy, as evidenced by increased c-fos expression (Calisi et al., 2016). Therefore, the expectation was to observe a decrease in GnIH expression in this brain area during incubation. However, no difference in the number of GnIH mRNA-expressing cells in the LHy was found between groups in this study. Unfortunately, GnIH-immunoreactive cells could not be quantified in this area due to tissue damage. Taking into account the discrepancies between the number of GnIH mRNA-expressing and GnIH-immunoreactive neurones in the PVN, it is possible that the hybridisation procedure used in this study was not sensitive enough to detect subtle differences in expression. It is also possible that the mechanism through which GnIH affects feeding in the LHy does not involve any changes in the number of neurones but only their activation. As no experiments have been conducted measuring c-fos expression coupled with GnIH administration and feeding in adult birds, it is not known whether the same LHy neurones would have been activated in adults as in chicks (Calisi et al., 2016). As shown in the sheep, while GnIH and its orthologues usually have orexigenic effects, GnIH also stimulated the activity of anorexigenic POMC cells in this species (Clarke et al., 2012). Thus, the possibility exists that GnIH may have an anorexigenic effect in the context of incubation. These data suggest that there may be a difference between the effects of GnIH in chicks and adults, or the orexigenic actions of GnIH are blocked in incubating birds through a mechanism which is unclear at present. Studies on the effects of GnIH on the feeding behaviour and c-fos expression of adult chickens are necessary to determine whether the orexigenic actions of this peptide change with sexual maturation. In addition, future work should focus on determining the exact involvement of each brain nucleus in the GnIH-mediated control of feeding. An examination of c-fos expression from GnIH neurons in adults, as well as GnIH-immunoreactive cells in the LHy during incubation and in different feeding conditions could provide us with better understanding about how GnIH is involved in feeding.

Conclusion

To the author's knowledge, this is the first study on the changes in GnIH throughout the entire reproductive cycle, from egg-laying through to the first day of chick-rearing, in a precocial bird species. The data presented in this chapter suggest that GnIH neurones in the PVN play a role in the control of incubation behaviour in the domestic hen. It is unclear at present whether GnIH has any role in the rearing of chicks in chickens as it seems to in songbirds. In order to determine whether this peptide has a function in rearing, studies involving the inhibition of the GnIH system through various means including the use of antagonists, gene knockout or silencing coupled with observations on the effects of these treatments on maternal behaviour in the presence of chicks can be conducted. In addition, double-labelling for GnIH and an indicator of neuronal activation such as the immediate early gene c-fos will show whether the presence of chicks has an effect on the GnIH system. As this system has shown plasticity in rats and starlings in response to changes in environment including loss of progeny, similar studies should be conducted to determine whether the same plasticity is present in precocial bird species.

No evidence was found in this study that GnIH is involved in the control of feeding behaviour in the LHy of incubating hens. However, GnIH immunoreactivity in this brain area could not be quantified and neuronal activation was not measured. This should be addressed in future work with studies at different times throughout the reproductive cycle, as well as in artificially food-restricted birds at different ages, to help confirm the possible role of GnIH neurones in this area in feeding in avian species and determine whether a difference exists between young chicks, juveniles and adults.

The findings of this study present for the first time an overview of the changes in the GnIH system in the brain of a precocial bird – the domestic hen. They contribute to our knowledge of the roles of GnIH in different species which are still not completely understood and highlight a possible difference in its functions between altricial and precocial bird species.

Chapter 6 Prolactin and Monoamines Throughout the Reproductive Cycle of the Domestic Hen

Abstract

Prolactin is involved in maternal care in mammals and birds and it is important in incubation and rearing. It is known to be controlled by the dopaminergic system through the D1 dopamine receptor (D1, D1R) which promotes and the D2 dopamine receptor (D2, D2R) which inhibits its secretion. It has been shown in the turkey that D2 mRNA expression is inversely correlated with prolactin mRNA expression in the pituitary gland, but this has not been examined in the chicken or in birds rearing chicks. In this study, qPCR was used to examine the mRNA expression levels of prolactin and D2 and their possible relationship at different time points in the domestic hen's reproductive cycle. The results presented in this chapter did not confirm a relationship between D2 and prolactin in the pituitary gland, as they showed no significant difference for either prolactin or D2 between any of the examined reproductive stages, suggesting that changes in prolactin and D2 mRNA expression in this gland are not crucial for the display of incubation and rearing. It is possible that other mechanisms contribute strongly to the increase in plasma prolactin during incubation. Methods measuring immunoreactivity as well as mRNA expression and techniques utilising D2 agonists and antagonists should be applied in future studies to more fully examine the relationship between the pituitary dopaminergic system and prolactin.

Apart from controlling prolactin, dopamine is also involved in maternal and other social behaviours, along with other monoamines including serotonin (5-hydroxytryptamine, 5-HT), noradrenaline and adrenaline. Dopamine and 5-

HT are part of the reward system, and motherhood has been shown to be rewarding to the mother. These monoamines are present in the raphe nucleus but, to the author's knowledge, the changes in their levels throughout the reproductive cycle from egg-laying through to chick-rearing have not been characterised in birds. Liquid chromatography - mass spectrometry (LC-MS) was used to examine the concentrations of monoamines in the raphe nucleus throughout the reproductive cycle in the domestic hen and test the hypothesis that they might be involved in maternal care in this species. No differences were found between groups for any of the compounds examined which included adrenaline, noradrenaline, dopamine, 5-HT, the dopamine precursor dihydroxyphenylacetic acid (DOPAC), the 5-HT precursor tryptophan and tryptophan's metabolite hydroxyanthranilic acid. These results do not support a role for monoamines in incubation or rearing behaviour in the chicken but further studies on monoamine release, rather than only content in the raphe nucleus, during the reproductive cycle can be used to better test this hypothesis.

6.1 Introduction

6.1.1 Prolactin

As established in Chapter 1, prolactin is a hormone secreted by the anterior pituitary gland involved in a number of physiological and behavioural functions in both birds and mammals, including metabolism, stress, lactation in mammals, and incubation in birds. Its effects are complex and varied. It has been demonstrated that prolactin can act as an insulin antagonist in dogs (Sinkoff and Bodo, 1953), and in the pigeon, it increased production, deposition, and turnover of fatty acids (Goodridge and Ball, 1967), showing

its involvement in metabolic processes. In the chicken, administration of low doses of prolactin increased the fractional excretion of sodium and chloride, suggesting that prolactin may have a role in avian osmoregulation (Roberts, 1998). Prolactin concentrations vary with the reproductive cycle, and it is an important regulator of reproduction. In the white-throated sparrow, it was found that there was a diurnal rhythm in the release of prolactin which changed seasonally - it was at noon in May and at midnight in August - and this diurnal release was suggested to be an important seasonal regulator of physiological and behavioural conditions in this species (Meier et al., 1969). In the ovaries in the chicken, prolactin, through its receptor in granulosa cells can have both pro- and anti-gonadal functions (Hu et al., 2017). In male geese, the period of lowest sperm quality coincided with the highest plasma prolactin levels, suggesting that prolactin has anti-spermatogenic functions (Gumulka and Rozenboim, 2015).

6.1.1.1 Prolactin in parental behaviour

6.1.1.1.1 Prolactin in pregnancy and lactation

Prolactin in mammals is known for its role in pregnancy and lactation (Bradley and Clarke, 1956; Kalyani et al., 2017; Meites and Sgouris, 1954). Prolactin injected into the mammary glands caused milk production in the rabbit (Bradley and Clarke, 1956). The secretion of prolactin itself was shown to be facilitated by suckling, as in lactating rats separated from their pups, plasma prolactin decreased to the same concentrations as in virgins as soon as 1 hour after pup separation (Kalyani et al., 2017). The mammary response to prolactin can be regulated by gonadal steroids as demonstrated in the

rabbit, where it was inhibited by estrogen and progesterone in combination (Meites and Sgouris, 1954; Meites and Sgouris, 1953).

6.1.1.1.2 Prolactin in incubation

In birds, prolactin is vital for the onset and maintenance of incubation, as demonstrated by numerous studies in various avian species. In the turkey, prolactin levels were highest during incubation, and they were higher in laying than in non-laying turkeys (Cherms et al., 1962). Similarly, plasma levels in the duck rose rapidly in the days before starting incubation and appeared to be closely correlated with time spent in the nest (Hall, 1991). Stimuli from the eggs have been shown to induce an increase in prolactin in chickens (Zadworny et al., 1988). There is a strong relationship between prolactin, incubation and energy balance, as evidenced by the finding that in eiders, when incubation started prolactin levels were correlated with body mass, suggesting that presence in the nest is determined by the female's initial body condition (Criscuolo et al., 2006).

As mentioned previously, prolactin has been shown to be responsible for the formation of the incubation patch in various birds (Höhn and Cheng, 1965), and this appears to be facilitated by gonadal steroids, as in non-breeding California quail, daily injections into the pectoral muscle of incubating birds of prolactin together with any gonadal steroid produced defeathering, epidermal hyperplasia, vascularisation and edema, while prolactin on its own only produced epidermal hyperplasia (Jones, 1969). In the same study, prolactin caused complete incubation patch formation in breeding birds (Jones, 1969). Estrogen, in combination with prolactin or progesterone, delivered through injections into the pectoral muscle, also produced the incubation patch in ovariectomised canaries (Hutchison et al., 1967). However, prolactin is equally important during incubation even in birds which do not form an

incubation patch. In the cape gannet where both sexes incubate a single egg with their feet and don't have an incubation patch, prolactin was higher in both sexes when they were incubating compared to in non-breeding birds (Hall, 1986). There is also evidence in the snow petrel that egg neglect was negatively correlated with plasma levels of prolactin (Angelier et al., 2015), and in the common tern, at the individual level, high prolactin during incubation was correlated with food abundance and it predicted not only better hatching but better fledgling (Riechert et al., 2014).

6.1.1.1.3 Prolactin in the care of chicks

As in mammals, where it has important functions after birth, the role of prolactin in birds appears to go beyond incubation. In the biparental zebra finch, in both sexes, plasma concentration of prolactin was significantly elevated both in late incubation and early post-hatch, compared to non-breeding, and in agreement with results from other species, this elevation was even higher in reproductively experienced birds (second breeding cycle), compared to birds breeding for the first time (Smiley and Adkins-Regan, 2016). Higher baseline prolactin in mourning doves was also correlated with higher nestling weight at the early stages (Miller et al., 2009). In the Manx shearwater, prolactin was high throughout chick rearing and was only slightly lower in late chick rearing, in failed breeders, and in non-breeders than in successful breeders (Riou et al., 2010). In the long-lived black-legged kittywake, chick-rearing birds (parental effort) compared to failed breeders (no parental effort) had higher prolactin, and the decrease in prolactin which occured after a stressor in these and many other bird species, was smaller in rearing birds, compared to failed breeders (Chastel et al., 2005). The link between prolactin, stress, and parental behaviour is discussed further in the next section.

6.1.1.2 Prolactin and the stress response in non-parental and parental animals

Prolactin is known to be involved in the stress response although the direction in which plasma levels of this hormone vary is context-dependent and possibly varies between species. Handling stress, anaesthesia, and blood sampling increased prolactin in male rats (Euker et al., 1975), and in humans, prolactin was elevated by various stress conditions including surgery and exercise (Noel et al., 1972). However, in ovariectomised estrogen-treated female rats, while prolactin increased consistently in response to restraint stress in the morning, in the afternoon the response depended on the already present prolactin level - if it was low response was as in the morning but if it was high the response was reversed and prolactin levels decreased (Smith and Gala, 1977). In the same study, there was no response in ovariectomised rats not treated with estrogen, which suggests that estrogen is necessary to mediate this response (Smith and Gala, 1977). There were similar findings in birds. In the turkey, acute restraint stress elevated plasma prolactin (Le Halawani et al., 1985), but it decreased it in the rock pigeon (Angelier et al., 2016). In a sea bird - the Manx shearwater - prolactin levels decreased in response to acute stress during incubation and mid chick-rearing but increased with stress during late chick-rearing and in non-parental birds, which is similar to the pattern observed in mammals (Riou et al., 2010). Indeed, the prolactin stress response and its relationship with the stress response mediated by the stress hormone cortisol (corticosterone in birds, CORT) is particularly relevant in parental animals. Prolactin likely contributes to the attenuated stress response during lactation in some mammals, as in lactating rats, stress-induced HPA activity was reduced but returned to normal after 24 h of separation from the pups when both plasma

prolactin and the expression of its receptor (PRLR) in the brain were back to the same levels as in virgins (Kalyani et al., 2017). Prolactin levels are often connected to those of corticotropin releasing hormone (CRH). A study in mice showed that CRH mRNA decreased in pregnant and nursing mice, compared to virgins, but removal of the pups, and therefore the suckling-induced prolactin increase, restored CRH levels. However, when females with the pups removed were treated with prolactin through an osmotic minipump, this again suppressed CRH (Gustafson et al., 2017).Nevertheless, it is important to note that in the same study, suppression of prolactin in lactating mice did not affect CRH, so while prolactin appeared to trigger the CRH decrease it did not seem to be necessary to maintain it (Gustafson et al., 2017). In addition, in the rat and mouse, CRH neurones in the PVN were not found to express PRLR, which suggests that the HPA-axis regulation by prolactin is not achieved through direct effects on CRH neurones (Gustafson et al., 2017). Similar to mammals, in many bird species, the stress response is attenuated during the parental period and this phenomenon may be regulated by prolactin. In the long-lived black-legged kittiwake, chick-rearing birds showing parental effort had higher baseline prolactin, as well as higher baseline CORT, compared to failed breeders, and while the CORT response to stress was unchanged, the stress-induced decrease in prolactin in rearing birds was significantly smaller, compared to that in failed breeders (Chastel et al., 2005). It was suggested in that study that the preserved elevation of CORT with stress caused foraging in order to ensure individual survival while the lack of decrease in prolactin ensured parental behaviour was still present (Chastel et al., 2005). In cape petrels, prolactin plasma concentrations were also higher, and the prolactin stress response was smaller in successful compared to failed breeders (Angelier et al., 2013). In snow petrels, older females who were less likely to neglect their eggs had an increased attenuation of the prolactin stress response, as evidenced by smaller decreases in prolactin concentrations after stress, compared to younger females (Angelier et al., 2007). In Eurasian hoopoe females, the stress-

induced prolactin decrease was smaller in early rearing when the young needed more care than in late rearing (Schmid et al., 2011).

6.1.1.3 Regulation of prolactin by the dopaminergic system

Previous studies have shown that the dopaminergic system controls prolactin in incubation. Tyrosine hydroxylase (TH) is often used as a marker for dopaminergic neurones, and in the native Thai hen, both plasma prolactin and TH-immunoreactive neurones in the nucleus intermedialis (nI), which is one of the nuclei responsible for regulating the VIP/prolactin system, were higher in rearing hens than in non-rearing (chick-deprived) hens in the first 14 days of rearing (Chokchaloemwong et al., 2013). As demonstrated by a number of studies in the turkey and chicken, the action of dopamine is achieved through its receptors D1 (D1D) and D2 which are stimulatory and inhibitory, respectively. In bantam hens, dopamine binding sites in the anterior pituitary were shown to decrease in incubating birds (Macnamee and Sharp, 1989). In addition, in the turkey, D1 was strongly expressed in the hypothalamus while D2 was more strongly expressed in the pituitary gland, and D1 was higher in the anterior hypothalamus, POM, infundibular nucleus (INF) and VMH of laying hens, compared to non-photostimulated hens, as well as in the INF of incubating hens, compared to laying hens (Chaiseha et al., 2003). In the same study, it was found that during the photorefractory stage which coincided with hypoprolactinemia, D1 was at its lowest everywhere in the brain while D2 in the INF increased (Chaiseha et al., 2003), and another study discovered that blocking D1, as well as immunisation against VIP, prevented the stimulatory effect on prolactin of infusions of dopamine into the brain (Youngren et al., 1996). As further evidence for the role of these receptors in the control of prolactin, both D1 and D2 were colocalised with VIP-expressing neurones in the lateral

hypothalamus and INF (Chaiseha et al., 2003). In the pituitary, while D2 decreased significantly during laying and incubation when prolactin concentrations were higher, compared to non-photostimulated and photorefractory birds (Chaiseha et al., 2003), D1D (a form of D1) expression increased significantly during laying and incubation (Schnell et al., 1999). It has been suggested in the turkey that dopamine acting on its D2 receptor in pituitary cells inhibits the stimulatory effects of VIP on prolactin release (Youngren et al., 1996). This likely happens through Ca^{2+} channels, as both a Ca^{2+} channel activator and VIP increased Ca^{2+} in cells, while both a channel blocker and a D2 agonist decreased Ca^{2+}. Similarly, both the channel blocker and the D2 agonist diminished the stimulatory effect of VIP on prolactin expression and release (Al Kahtane et al., 2005).

6.1.2 Monoamine neurotransmitters

Dopamine, serotonin (5-HT), adrenaline, and noradrenaline are monoamine neurotransmitters derived from aromatic amino acids which possess an amino group and an aromatic ring (Mele et al., 2010). As noted in Chapter 1, they are synthesised in the brain and play roles in cognition, behaviour, memory and stress, but of interest for this study is their role in maternal behaviour.

6.1.2.1 Involvement of monoamines in maternal behaviour

Previous studies have shown that dopamine and noradrenaline play a role in the control of maternal behaviour. Lesions in the rat brainstem which depleted noradrenaline in the cortex and hippocampus resulted in deficits in

the onset of maternal care in primiparous rats, suggesting that noradrenaline is important for the onset of this behaviour (Steele et al., 1979). In agreement with these findings, noradrenaline depletion before parturition in rat dams, achieved through intraventricular infusion of the noradrenaline antagonist 6-OHDA, also had a negative effect on the onset of maternal behaviour, but not on its maintenance once pups were born (Rosenberg et al., 1977). In mice, lesions to the central noradrenergic projections to the olfactory bulbs before parturition lead to cannibalism, although they did not otherwise impair maternal behaviour, and they had no effect after parturition. These results suggested that the noradrenaline system was important for maternal recognition in this species (Rothlin et al., 1922). Further supporting this hypothesis, mice with 6-OHDA-induced lesions to the medial olfactory stria which led to the depletion of noradrenaline in the olfactory bulbs resulted in a failure to recognise pups at parturition (Calamandrei et al., 1992). In rat dams, during gestation, inhibiting the noradrenaline transporter or both the noradrenaline and 5-HT transporters lead to reduction of maternal behaviour, and in high doses, also to the reduction of aggression (Cox et al., 2011).

In addition to 5-HT and NA, dopamine is also known to be important for maternal care. Single and repeated IP injections of a D2 antagonist in albino rat attenuated parental care as soon as 20 min post injection, with chronic injections on days 1-6 postpartum having a more pronounced and stable effect (Tanaeva et al., 2012). Also in the rat, infusion with dopamine receptor antagonists for both D1 and D2 before parturition led to impaired maternal care immediately after birth, and the D2 antagonist also led to impaired retention of maternal care when rats were tested 7 days later (Byrnes et al., 2002). Thus, while both receptors seem to be involved in immediate care, results suggest that D2 also plays a role in the retention of the behaviour. Furthermore, in a strain of mice which naturally displayed maternal neglect, it was discovered that neglectful mothers had elevated c-fos expression in dopamine-related areas of the brain like the zona incerta (ZI), arcuate

nucleus and nucleus accumbens, and TH was significantly elevated in ZI. Phosphorylation of the compound DARPP-32 - a marker of D1 activation - was also higher in nucleus accumbens and the caudate-putamen nucleus of neglectful mothers (Gammie et al., 2008). These results together suggest that atypical dopamine activity in regions involved in reward may be related to maternal neglect. Similarly, the hubb/hubb mutant mouse which showed impaired maternal behaviour had higher levels of the dopamine metabolite DOPAC in the median eminence in early lactation than in the wild type control mouse (Alston-Mills et al., 1999). Interestingly, the dopamine system appears to also control maternal behaviour through its involvement in interactions between the mother and the young. In rats, exposure to ultrasonic vocalisations (USV) emitted by pups increased plasma prolactin in wild type but not D2 knockout dams, who also showed delayed pup retrieval and nest building, and D2 knockout rat pups emitted fewer USV suggesting that both D2 and pup-dam genotype interactions are important for maternal care (Curry et al., 2013). As further evidence for the importance of the dopaminergic system in mother-pup interaction, in rat pups when dopamine in the striatum was depleted via 6-OHDA administration, maternal care displayed by their mother was impaired (Wilkins et al., 1997). The involvement of 5-HT in the raphe nucleus in maternal care is further discussed in Chapter 7.

6.1.3 Aim and hypothesis

The overal aim of this study was to examine the possible involvement of prolactin and the dopaminergic, serotonergic, adrenergic and noradrenergic systems in the brain of the domestic hen in incubation and rearing. One specific aim was to test whether a decrease in D2 receptor expression could be observed in the chicken pituitary gland during incubation as has been

previously observed in the turkey, and whether this decrease corresponded to an increase in prolactin expression in this gland during the same reproductive stage. The possible changes of the concentrations of monoamines in the raphe nucleus throughout the reproductive cycle of the chicken had not been studied prior to this project but monoamines had been found to be involved in maternal behaviour in previous studies. Therefore, the hypothesis that dopamine and 5-HT content in the raphe nucleus would be elevated with the display of maternal behaviour, along with norarenaline, while adrenaline could be downregulated in relation to a possibly attenuated stress response in maternal chickens, was also tested.

Hypothesis:

Based on previous studies cited above, it was hypothesised that prolactin mRNA expression in the pituitary gland of the domestic hen would increase with the onset of incubation while expression of the D2 receptor decreased. It was also hypothesised that dopamine, 5-HT and noradrenaline content in the raphe nucleus would increase during incubation and rearing, supporting the involvement of these monoamines in maternal care, while adrenaline content decreased in relation to a possibly lowered stress response.

6.2 Materials and methods

6.2.1 Animals and housing

The animals used in these experiments, as well as the experimental design and housing conditions, were the same as already described in Chapter 3.

6.2.2 Tissue collection

Brains and pituitaries were collected as described in Chapter 3. Both lobes of the pituitary (anterior and posterior) were collected and analysed together as their separation is practically impossible in birds (Ritchie and Pilny, 2008). A block of tissue rostral to the pons containing the dorsal and median midbrain raphe nuclei (referred to as “the raphe nucleus” throughout this chapter) was later dissected out from the frozen brains with the help of a scalpel and kept frozen until being processed as described further down. The pons and the cerebellum were used as indicators of the position of these nuclei – a cube of tissue at the level of the pons and before the cerebellum was dissected out.

6.2.3 Quantitative polymerase chain reaction (qPCR)

A general introduction to this procedure can be found in Chapter 2.

6.2.3.1 RNA extraction and purification with Direct-zol

A Direct-zol RNA Miniprep Kit including TRI reagent (Zymo/Cambridge Bioscience) was used in the procedures described below. 500µl TRI Reagent was added per 50mg tissue. Ultra Turrax homogenizer was used to homogenise the tissue in a 1.5ml Eppendorf tube. Samples were homogenised in short bursts (approx 10 sec. at speed 6) and the homogeniser probe was washed in ddH_2O (two 50ml Greiner tubes, wash 1 and wash 2) before each sample. After homogenisation was complete, each sample was centrifuged in a standard bench top centrifuge at 13 000rpm for 1 minute. The supernatant was transferred to an RNase-free tube. All centrifugation steps were performed at top speed. One volume of 100% ethanol was added to every one volume of sample homogenate in TRI Reagent. The mixture was vortexed thoroughly and then transferred to a Zymo-Spin column in a Collection Tube and centrifuge for 1 minute. Column was transferred into a new Collection Tube and the flow-through was discarded.

At this point RNA samples were in-column treated with DNase 1. The column was washed with 400 µl RNA Wash Buffer and centrifuged for 30 seconds. Flow-through was discarded. A DNase 1 reaction mix was prepared in an RNase-free tube for each separate sample. 5 µl of DNase 1 (1 U/µl), followed by 75µl DNase Digestion Buffer were added to the tube. This mix (80µl) was added to the column matrix and the column was incubated at RT for 15 minutes before being centrifuged for 30 seconds. After this treatment, 400 µl

Direct-zol RNA PreWash were added and the column was again centrifuged for 1 minute. Flow-through was discarded. This step was repeated one more time. 700 µl RNA Wash Buffer were added to the column and it was centrifuge for 1 minute. Flow-through was discarded. The column was centrifuged for an additional 2 minutes in an emptied Collection Tube to ensure complete removal of buffer. The column was then transferred to an RNase-free tube and 50µl of DNase/RNase-Free Water were added directly to the column matrix. The column was centrifuged for 1 minute. Optical density/concentration of purified sample was measured with Nanodrop.

6.2.3.2 Reverse transcription of purified RNA into cDNA

High Capacity Reverse Transcription Kit (Applied Biosystems) was used in the procedures described below according to the manufacturer's protocol. 1µg purified RNA (in 10µl RNase-free water) per sample was put into a PCR strip tube. 2µl RT buffer, 0.8µl dNTP, 2µl random primers, 1µl reverse transcriptase and 4.2µl H_2O were added per sample. A master mix was prepared for all samples and then added to the strip tubes, while pipetting up and down to mix. Thermal conditions were 25˚C for 10 minutes, 37˚C for 120 minutes, 85˚C for 5 minutes and then cooled to 4˚C to hold. After the program had run, 90µl MilliQ H_2O were added to each sample to make a final volume of 110µl. The resultant cDNA was stored at -20˚C until use.

6.2.3.3 PCR to test primers and generating product for standard curve

A number of previously designed primer pairs were tested to confirm the primers only amplified the desired sequence and determine which pair gave the best amplification. Agilent Brilliant III Ultrafast SYBRgreen qPCR mix was used for this procedure. For all primers, PCR with SYBRgreen was run on regular PCR machine using the same program as used on mx3000 for qPCR and product was run on 1% agarose gel to confirm the presence of a single band. PCRs for standard curve generation were run on the same gel. The mix for each sample contained 10.0µl 2x Sybr mix, 0.3µl reference dye diluted to 1:500, 0.4µl each of the forward and reverse primer and 0.9µl sterile ddH_2O. To this 12µl mix, 8µl cDNA was added for a total of 20µl per well. To generate material for a standard curve, PCR was run using faststart Taq (Roche) on stock cDNA (pooled chicken pituitary cDNA) diluted 1:40. Each reaction contained 2µl 10x NTP mix, 2µl 10x buffer (faststart and Mg^{2+}), 0.1µl of 100µM primers 1 and 2, 13.7µl H_2O, 0.1µl FastStart Taq (Roche) and 2µl cDNA (pooled chicken pituitary cDNA from various samples, 1:40 dilution). Thermal conditions were 95°C for 4 min, 95°C for 30 sec repeated x40 cycles, then 58°C for 30 sec, 72°C for 30 sec and 72°C for 7 min. Products were run on 1% agarose gel. The band was cut out and purified with Qiagen gel extraction kit using the manufacturer's protocol. The amount of product was quantified with nanodrop. The product was sequenced using LightRUN Sanger sequencing (GATC Biotech) to confirm it was the correct sequence.

To generate a standard curve the stock obtained from the purified gel band was first diluted 1:10 and then this new stock was serially diluted to obtain concentrations of 1:500 to 1:5 000 000 000. These 8 dilution points were run on the qPCR machine as described below and the best 6-point range was selected to be used in the assay.

The primers used for prolactin were:

Forward: AGCTCAGCAGATTCATCATGA

Reverse: GCCTCTTGTTTTGCTCCTCA

And those used for D2 were:

Forward: TGCACTCATCCAGAAGACGT

Reverse: GGCTTGGCAAGTGTTCTCTC

6.2.3.4 qPCR reaction

qPCR was used in this case because this procedure is quick, safe, and can be repeated many times with very small amounts of tissue. This made it very suitable for measuring gene expression, which was the goal. The small size of the pituitary glands collected would have made the use of ISH extremely difficult and, as the distribution of mRNA expression for prolactin and D2 in the pituitary was not of particular interest in this study, it would have been unnecessary, especially considering its cost, along with health and safety considerations.

Samples were run in a 96-well detection plate with optical caps ABI on an MxPro3000 rtPCR machine (Stratagene) along with the standards generated above. Thermal conditions were 50˚C for 2 min, 95˚C for 2 min, 95˚C for 15

sec repeated x40 cycles, then 60˚C for 30 sec, 95˚C for 1 min, 60˚C for 30 sec, 95˚C for 15 sec and then cool to 4˚C and hold. Samples and standard curve were run in duplicate. A blank and positive control (pooled chicken pituitary cDNA diluted 1:40) were run in triplicate.

6.2.3.5 Normalisation and analysis

The gene expression level for each sample in the reaction was calculated using a standard curve. Results for both prolactin and D2 for each sample were normalised to the geometric mean of results for two reference genes - LBR and YWHAZ - which have been previously demonstrated to be reliable in chicken. This method has been used previously by Reid *et al* (Reid et al., 2017).

The primers used were:

For LBR:

Forward: GGTGTGGGTTCCATTTGTCTACA

Reverse: CTGCAACCGGCCAAGAAA

For YWHAZ:

Forward: GTGGAGCAATCACAACAGGC

Reverse: GCGTGCGTCTTTGTATGACTC

qPRC reactions for these two genes were run for all samples under the exact same conditions as above. As only the difference between groups and not actual expression was of interest, for better clarity, the data are presented in arbitrary units where the average of all results from the Laying group is taken to equal 1 and each result is calculated as a fraction of this number. The regressions curves had an R^2 value above 0.996 for all reactions.

6.2.4 Liquid Chromatography Mass Spectrometry (LC-MS)

6.2.4.1 Buffers and standards

Homogenisation buffer consisted of 20mM ascorbic acid while labelling buffer consisted of 20mM sodium tetraborate. The labelling reagent was 2% benzoyl chloride in acetonitrile. The dilution buffer for the standards was and 1mM ascorbic acid in equal volumes of water and methanol. Standards were prepared to 1mg/ml in homogenisation buffer. 25µl of each standard solution were mixed in an Eppendorf and diluted to 25µg/µl. Standard were then serially diluted in homogenisation buffer to 10µg/ml, 2.5µg/ml, 500, 125, 31.25, 7.8, 1.9, 0.5 ng/ml. 25µl caffeic acid stock solution was diluted in 975µl homogenisation buffer to get 25mg/ml.

6.2.4.2 Homogenisation, labelling and analysis of samples

Samples were homogenised in 100µl homogenisation buffer for every 50mg of brain tissue and divided into 100µl aliquots which were kept at -70°C until use. On the day of the procedure samples were thawed and 2µl caffeic acid solution was added to each to achieve a final concentration of 500ng/ml. 500µl ice cold acetonitrile were also added and the samples were vortexed and sonicated several times each for up to 20 seconds. The same was done with all standards. All samples were centrifuged for 5min at RT at top speed to remove precipitate. Supernatant was dried in freeze dryer overnight. Dried samples were stored at -20°C until use.

25µl labelling buffer and 25µl labelling reagent was added to the dry supernatant and incubated at RT for 5 min. Samples were centrifuged to remove particulates for 5 min at top speed and then analysed by LC-MS. The column contained Kromasil c18, 2.1mm id, 150mm length. The solvents consisted of 0.1% formic acid, 2mM ammonium acetate in water and acetonitrile. Flow rate was 0.2ml/min. Gradient was 30% B for 1.5 minutes, to 65% B over 2 min, held at 65% B for a further 1.5 min, to 75% B at 8 min then 30% B at 10 minutes for re-equilibration. The method used has been previously described (Ritchie et al., 2002).

6.2.4.3 Analysis of LC-MS results

Samples were run in two batches of 16 samples each, and each sample was run in duplicate for every assay (two technical replicates). Each batch was run twice and the average of the results from the two runs was used for all

compounds with the exception of tryptophan and noradrenaline where results from a second run were not available so only one run was used.

The average intra and inter-assay variability for each compound, as well as the leniarity and threshold of detection is shown on Table 6.1. The inter-assay variability was very high. However, only the difference between groups was of interest, rather than actual concentrations, so in order to overcome this, the average of all samples in the laying group was taken to equal 100%. Individual values were calculated as % of this average. These were also the values used in the calculation of the inter-assay variability as they were believed to be a more accurate representation of the variability of the results presented in this thesis. However, the original values were used in calculating the intra-assay variability. Once again, because only differences between groups were of interest, controls for the recovery rate and assay accuracy were not deemed necessary.

As the coefficients of variation were fairly high and inter-assay variability could not be obtained for some samples, results should be interpreted with caution. Tryptophan was the only compound with low average intra-assay variability (%CV was 2.722) but this was obtained from one run only. For all other neurotransmitters both the intra- and inter-assay variability was above 20.

Table 6.1 Quality controls for LC-MS Monoamine Neurotransmitters Assay

Analyte	Intra-assay variability (%CV)	Inter-assay variability (%CV)	Linearity	Limit of detection (ng/ml)
Dopamine	38.178	33.401	0.992	0.488
DOPAC	31.802	21.636	0.996	0.488
Serotonin	31.178	22.243	0.993	0.488
Tryptophan	2.722	n/a	0.997	0.488
Hydroxy-anthranilic acid	21.612	22.308	0.991	0.488
Adrenaline	38.742	27.732	0.995	0.488
Noradrenaline	22.292	n/a	0.990	0.488

Figure 6.1 shows a representative chromatograph for the LC-MS assay with labelled peaks.

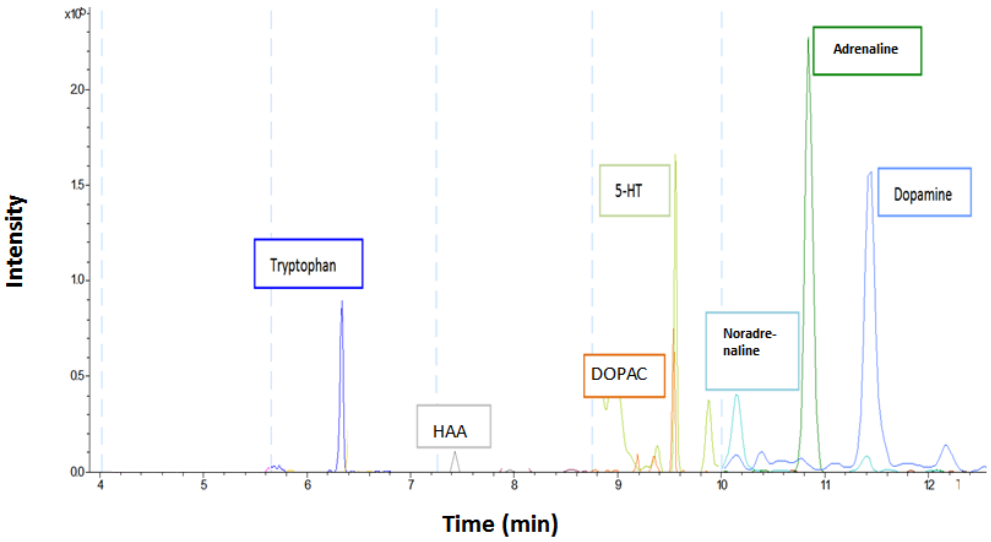

Figure 6.1 Representative extracted ion chromatogram using neurotransmitter standards prepared in homogenising buffer. Peaks are labelled with the compound name in a box of the same colour as the relevant peak. 5-HT – serotonin; HAA – hydroxyanthranilic acid; DOPAC – 3,4-dihydroxyphenylacetic acid.

6.2.5 Statistical analysis

Either One-way ANOVA or Kruskal-Wallis ANOVA on ranks was used to analyse the results. $P<0.05$ was considered statistically significant.

6.3 Results

6.3.1 Prolactin and D2R mRNA in the pituitary gland of hens throughout the reproductive cycle

There was no significant difference between groups in the expression of either prolactin (Kruskal-Wallis ANOVA on ranks, $H_{(3)}$=6.798, p=0.079) or D2 (One-way ANOVA, $F_{(3\text{-}23)}$=2.052, p=0.135). Results are shown on Figure 6.2.

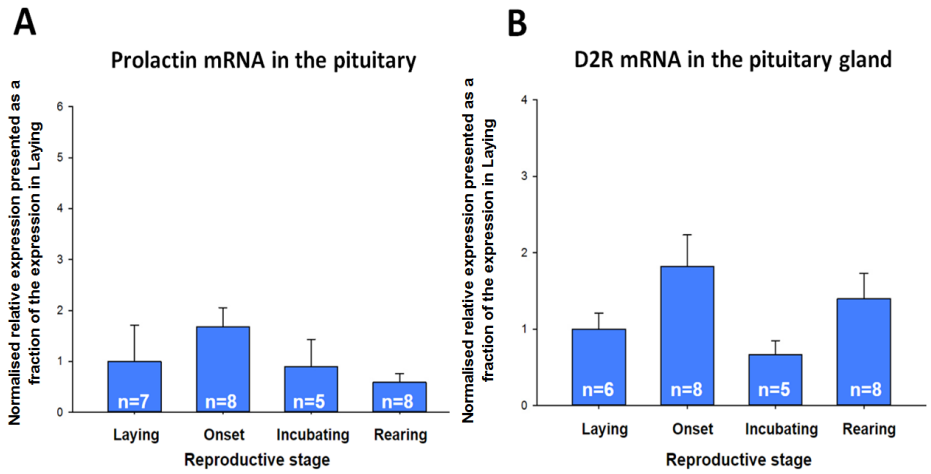

Figure 6.2 Prolactin and D2R dopamine receptor in the pituitary gland of the domestic hen throughout the reproductive cycle. Data are presented relative to layers with the average value for layers taken as 1. There was no significant difference between groups for either prolactin (Kruskal-Wallis ANOVA on ranks, $H_{(3)}$=6.798, p=0.079) or D2R mRNA expression (One-way ANOVA, $F_{(3\text{-}23)}$=2.052, p=0.135). n=5-8 for both prolactin and D2R. (8 birds per group were included in the procedure but individuals were excluded from analysis when the concentration of the compound of interest in the sample could not be calculated by the instrument.) Data are presented as the mean +SEM.

6.3.2 Monoamines in the raphe nucleus of hens throughout the reproductive cycle

There was no significant difference between groups in the concentration of dopamine (One-way ANOVA, $F_{(3\text{-}24)}$=1.987, p = 0.939) or DOPAC concentration (Kruskal-Wallis ANOVA on ranks, $H_{(3)}$=0.639, p=0.887). Results are shown on Figure 6.3.

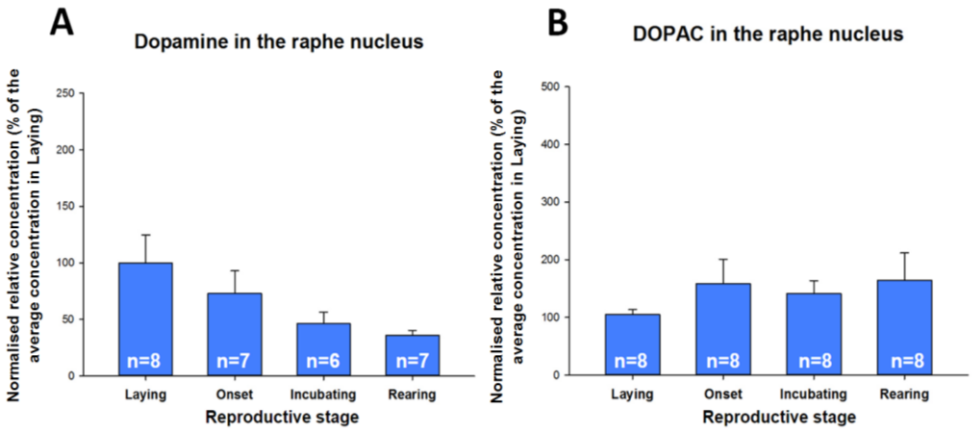

Figure 6.3 Concentration of dopamine and dihydroxyphenylacetic acid in the raphe nucleus of the domestic hen throughout the reproductive cycle.

Data are presented relative to layers with concentrations in layers taken as 100%. There was no significant difference between groups for either dopamine (One-way ANOVA, $F_{(3\text{-}24)}$=1.987, p=0.939) or DOPAC concentration (Kruskal-Wallis ANOVA on ranks, $H_{(3)}$=0.639, p=0.887) n=6-8 for dopamine and n=8 for DOPAC. (8 birds per group were included in the procedure but individuals were excluded from analysis when the concentration of the compound of interest in the sample could not be calculated by the instrument.) Data are presented as the mean +SEM.

Similarly, no difference was found between groups in this nucleus in the concentrations of 5-HT (One-way ANOVA, $F_{(3\text{-}23)}$=1.660, p = 0.203), its precursor tryptophan (One-way ANOVA, $F_{(3\text{-}25)}$=0.133, p = 0.939) or the tryptophan metabolite hydroxyanthranilic acid (One-way ANOVA, $F_{(3\text{-}28)}$=0.856, p = 0.475). Results are shown on Figure 6.4.

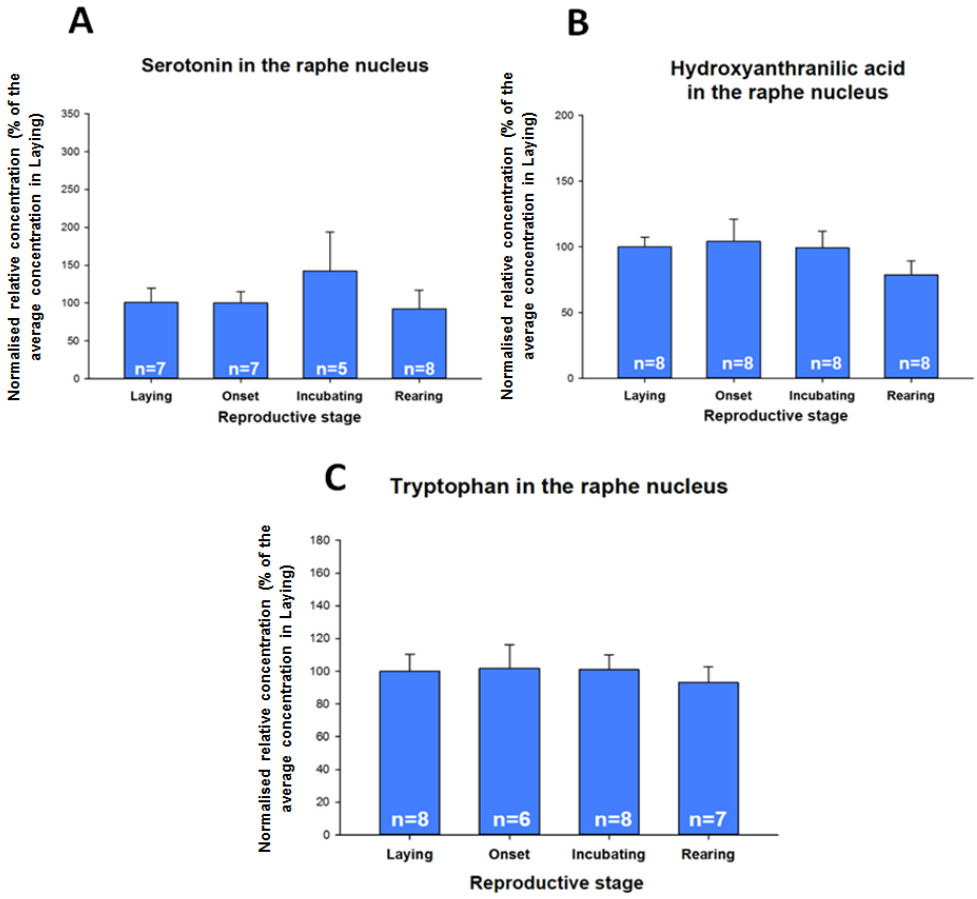

Figure 6.4 Concentration of serotonin, hydroxyanthranilic acid and tryptophan in the raphe nucleus of the domestic hen throughout the reproductive cycle.

Data are presented relative to layers with concentrations in layers taken as 100%.There was no significant difference between groups for 5-HT (One-way ANOVA, $F_{(3\text{-}23)}$=1.660, p = 0.203), tryptophan (One-way ANOVA, $F_{(3\text{-}25)}$=0.133, p = 0.939) or hydroxyanthranilic acid (One-way ANOVA, $F_{(3\text{-}28)}$=0.856, p = 0.475). n=5-8 for 5-HT, n=8 for hydroxyanthranilic acid and n=6-8 for tryptophan. (8 birds per group were included in the procedure but individuals were excluded from analysis when the concentration of the compound of interest in the sample could not be calculated by the instrument.) Data are presented as the mean +SEM.

Finally, there was no difference between groups in the raphe nucleus concentrations of (One-way ANOVA, $F_{(3\text{-}25)}$=0.462, p = 0.712) or noradrenaline (One-way ANOVA, $F_{(3\text{-}27)}$=0.739, p = 0.538). Results are shown on Figure 6.5.

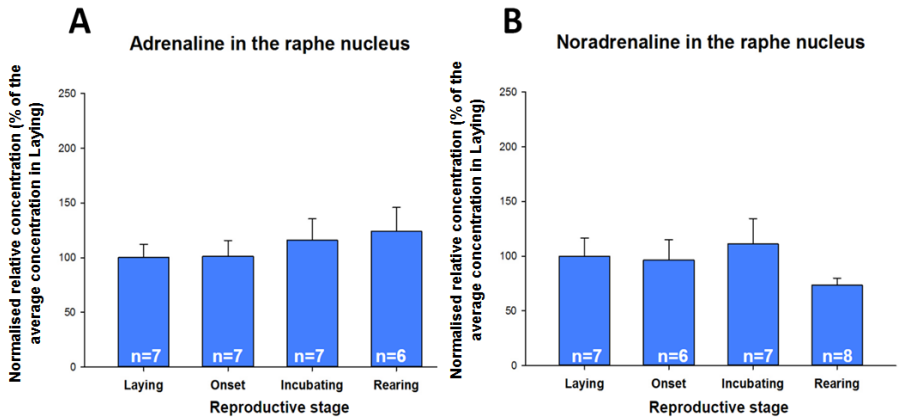

Figure 6.5 Concentration of adrenaline and noradrenaline in the raphe nucleus of the domestic hen throughout the reproductive cycle. Data are presented relative to layers with concentrations in layers taken as 100%. There was no significant difference between groups for either adrenaline (One-way ANOVA, $F_{(3\text{-}25)}$=0.462, p = 0.712) or noradrenaline (One-way ANOVA, $F_{(3\text{-}27)}$=0.739, p = 0.538) n=6-7 for adrenaline and n=6-8 for noradrenaline. (8 birds per group were included in the procedure but individuals were excluded from analysis when the concentration of the compound of interest in the sample could not be calculated by the instrument.) Data are presented as the mean +SEM.

7.4 Discussion

7.4.1 Prolactin and D2R in the pituitary gland throughout the reproductive cycle

An upregulation of prolactin during incubation has been documented in avian species. For example, higher prolactin content was measured in the anterior pituitary of incubating turkeys compared to laying and non-laying turkeys (Cherms et al., 1962), and an increase in plasma prolactin concentrations at the onset of incubation was found in ducks (Hall, 1991). However, most studies measured plasma prolactin concentrations or pituitary prolactin concentrations, and not prolactin mRNA expression in the pituitary gland. The fact that no differences were observed between the four examined reproductive stages while other studies have found a difference in plasma or pituitary concentrations could be explained by a high rate of translation in prolactin cells. No difference between groups was found in the expression of the D2 receptor either, even though this receptor has been shown to have an inhibitory effect on prolactin release in birds (Youngren et al., 1996), and it was found in bantam hens that dopamine binding sites in the anterior pituitary decreased during incubation (Macnamee and Sharp, 1989). However, the results presented here are in agreement with a previous study in the turkey which found that although D2 expression during both laying and incubation was significantly lower than in non-photostimulated and photorefractory turkeys, no significant difference was found in the expression of this receptor in the pituitary between laying and incubating turkey hens (Chaiseha et al., 2003). In addition, the present results do not show a change in D2 expression on the first day of rearing, compared to the laying period,

early, or later incubation, even though previous studies suggested that prolactin may be necessary for chick-rearing in some avian species (Chastel et al., 2005; Riou et al., 2010).

Together, the results presented in this chapter suggest that changes in D2 expression in the pituitary may not be crucial for the increase in plasma prolactin during incubation compared to laying. Plasma prolactin was not measured in the present study but its increase in incubation has been recorded in previous studies in the chicken (Sharp et al, 1987), turkey (Cherms et al., 1962) and duck (Hall, 1991). As both the stimulatory D1 and inhibitory D2 receptors have been found to be co-localised with VIP neurones in the lateral hypothalamus and INF (Chaiseha et al., 2003), it is possible that a downregulation of those receptors is necessary for the prolactin increase in incubating birds. A more detailed study of the expression of D1 and D2 in both the brain and pituitary of chickens throughout all reproductive stages is necessary. This should includethe non-photostimulated and photorefractory stages, as well as the stages examined here, and be coupled with measurements of plasma prolactin concentrations and immunoreactivity for both dopamine receptors and prolactin, and examination of the effects of dopamine receptor agonists and antagonists on prolactin would provide us with a better understanding of the relationship between DA-ergic receptors, prolactin and incubation in this species.

7.4.2 Monoamines in the raphe nucleus

No differences in the concentrations of any of the examined monoamines were observed between groups in this study. This fact may be due to species differences, as most of the data for their involvement in maternal care come from mammalian studies while little is known in birds. It has been shown that noradrenaline is important for maternal care in rats and mice, as a depletion

of its levels in the brain led to impairments in this behaviour (Calamandrei et al., 1992; Cox et al., 2011; Rosenberg et al., 1977; Rothlin et al., 1922; Steele et al., 1979). The raphe nucleus is one of the main areas of synthesis of this monoamine (Saavedra et al., 1976; Versteeg et al., 1976) so it was hypothesised that noradrenaline levels in the raphe nucleus would be increased with the display of maternal care. However, the results presented here revealed no difference in noradrenaline concentration in this brain region between any of the examined reproductive stages. This suggests that an increase in the raphe noradrenaline may not be necessary for the onset of maternal behaviour in the chicken.

It is true that the rearing hens in this study had only spent 24 hours with their chicks before tissue collection and, as described in Chapter 4, while all 8 of the birds in this group were displaying signs of maternal behaviour (gathering the chicks under their wings, clucking) only two of them had left the nest by the end of the experiment. A study in rats demonstrated that depletion of noradrenaline in the brain after pups were born did not interfere with maternal behaviour which is evidence against the possibility that a change in noradrenaline concentration might be observed after a longer time spent rearing (Rosenberg et al., 1977). In the case of dopamine, various studies have shown that antagonists to both the D1 and D2 dopamine receptors disrupt maternal care in its early stages (Byrnes et al., 2002; Tanaeva et al., 2012), suggesting that dopamine in the brain is involved in the behaviour, but no difference was found between groups in this study for either dopamine or its precursor DOPAC. Unlike noradrenaline, dopamine appeared to be involved in rearing after the initial onset of maternal care in previous studies (Byrnes et al., 2002; Tanaeva et al., 2012; Alston-Mills et al., 1999; Gammie et al., 2008), therefore, it is not unreasonable to suppose that an elevation of dopamine concentration may manifest later into the rearing period, especially since the dopaminergic system appeared to be important for interactions between the mother and the young in a study in mice (Curry et al., 2013).

The addition of a group which would have reared chicks for a longer period of time would provide information on the possible involvement of dopamine in the raphe nucleus in rearing after the initial stage.

5-HT originating from the raphe nucleus has been implicated in maternal care in rats, with high levels promoting the behaviour (Harding and Lonstein, 2016). However, the results presented here showed no difference between reproductive stages in the raphe nucleus concentration of 5-HT or its precursors tryptophan and hydroxyanthranilic acid. As with dopamine, it is possible that, while there was no measurable difference in concentrations on the first day of rearing, such a difference may appear after a longer period. As mentioned above, a study including a time point later in the rearing period could provide further information.

For all of the examined compounds, studies involving the delivery of agonists and antagonists to the brains of incubating and rearing chickens and specifically to the raphe nucleus would help determine whether they are of any importance for maternal care in this species. It would also be particularly interesting to investigate whether dopamine play any role in hen-chick interactions. This could be achieved through behavioural studies on hens with chicks coupled with the delivery of antagonists or the use of genetically engineered knockout chickens deficient for elements of the dopaminergic system.

Finally, it is possible that certain technical limitations of the procedure made it impossible to detect more subtle differences between groups. Due to husbandry issues, additional experimental birds could not be procured for this study. As a result, the brains of the hens used in the studies described in chapters 4 and 5 had to be used. Those brains were frozen and the larger part of them sectioned on a cryostat before dissecting the raphe nucleus out of the remaining chunk of tissue. This meant that the samples had gone through thawing to the temperature necessary for sectioning, re-freezing and

thawing once more before being used for LC-MS. In addition, while an effort was made to dissect the raphe nucleus accurately, the frozen state of the tissue at the time made this process difficult and some brain tissue not belonging to the nucleus of interest may have remained in the sample. It is possible that, despite attempts to ensure that samples were consistent, differences in the amount of this tissue may have interfered with results. In future studies, dissecting the raphe nucleus immediately after collecting the brain would make the process easier and would help eliminate this concern.

Conclusion

The results of this study fail to demonstrate a relationship between the expression of D2 and prolactin at the pituitary level in the chicken. While it is possible that this relationship is not present in this species, it is more likely that more sensitive techniques are required to observe it and measuring the levels of protein as well as mRNA may be beneficial. D2 receptor agonists and antagonists, as well as gene knockout and silencing could be used as well to see the effect on prolactin expression and concentrations. mRNA expression and immunoreactivity for the D1 receptor in both the pituitary and the hypothalamus should also be examined.

In the case of monoamines, the results presented here do not provide any evidence that they may be important for maternal behaviour in the raphe nucleus. However due to issues with the tissue and the reliability of the assay these results should be interpreted with caution. Further studies should include a better procedure to dissect the raphe out, better localisation of the raphe and better-quality tissue. In addition, the effects of antagonists for these monoamines or lesions in the chicken raphe nucleus on behaviour should be studied in the chicken in the context of maternal behaviour.

Knockout and gene silencing techniques can also be used in this case to further investigate the specific roles of monoamines and their receptors.

Overall, the results presented in this chapter underline the need for further studies on the regulation of the prolactin system in avian maternal behaviour as well as the serotonergic and other monoamine systems in the midbrain in birds.

Chapter 7 Activation of Brain Areas Relevant to Maternal Behaviour in the Adult Female Quail Following Interaction with Chicks.

Abstract

Maternal behaviour in many species of mammals and birds can be induced through the introduction of young pups or chicks to adult females. Interaction of individuals who are not mothers with young also occurs naturally and there is evidence that this experience may play a role in facilitating a quicker onset and better quality of maternal behaviour in later life. In rats, juveniles involved in the care of younger siblings later display better maternal behaviour than control animals not previously exposed to pups. For any parental behaviour to develop, it is necessary for an animal to distinguish between its adult conspecifics and young of the same species. Indeed, animals and humans display different behaviour towards young individuals compared to adults. Studying the differential response to young and adults in animals and the mechanisms which govern the switch from negative to positive responses to young is important for understanding the fundamentals of parental behaviour and the mechanisms which govern it.

As previously discussed, maternal behaviour is controlled in the brain by a number of brain nuclei. Neurones within the brain behaviour network, including in areas such as the PVN, MPOA/POM, SON, LS and BnST are activated during the display of maternal care and are responsive to stimuli from young. In addition, there are other areas which may be important for the behaviour. The brain raphe nucleus has also been implicated in the control of maternal behaviour through producing and releasing dopamine and serotonin which may be responsible for making motherhood rewarding to the mother. However, the expression of serotonin in the raphe nucleus can also be

induced by stress. The display of maternal behaviour is also accompanied by a downregulation of sexual behaviours as a result of the inhibition of gonadotropin-releasing hormone (GnRH). The nucleus of the commissurae pallii (nCPa) in the brain with its high density of GnRH neurones is one of the areas involved in this regulation. Based on recent studies, including data presented in this thesis, mesotocin appears to be one of the main hormones responsible for chick-rearing in domestic chickens and turkeys, therefore, it is likely that it also plays a similar role in other avian species. Further investigation into mesotocin is necessary to fully understand the mechanisms of its action and its role at different stages of the avian maternal response, and one of the important avenues for such investigation is examining the activation of mesotocinergic neurones with displays of maternal care. While studies involving the induction of maternal behaviour in virgin animals have been performed in both mammals and birds, avian studies have typically involved paradigms that required a lot of human intervention because of the use of very young chicks, and those studies have been primarily aimed at the development of full maternal behaviour. In order to map and quantify the brain regions activated following exposure to chicks, a study design was used which attempted to mimic natural cohabitation with younger siblings in order to determine whether this type of experience influenced the response to chicks of adult female quail. Adult females were introduced to a group of five 6-day-old chicks and allowed to cohabitate with them for six days. After this, the chicks were removed overnight and either a new group of chicks or a novel adult individual was introduced the next morning. The behavioural response of the experimental birds to the chicks or the novel adult was recorded and the activation of the behaviour system in the brain was analysed using c-fos immunohistochemistry. In order to determine if mesotocin neurones in the PVN were becaming activated in the presence of chicks, double-labelling for c-fos and mesotocin was also performed. The chicks used in this study were at least six days old and therefore could

survive well in a standard pen when provided with food and water without any extra care. It was found that the initial response of adult females was to actively avoid chicks, but this response changed by the sixth day of cohabitation, and when new chicks were introduced, females spent significantly more time with them than they did with a novel adult individual. Differences in the number of Fos-immunoreactive neurones were found between the two groups in the PVN, nCPa and the raphe nucleus while there was no difference in the POM, SON, LS and BnST. The PVN and nCPa of birds introduced to novel chicks had higher c-fos immunoreactivity than those introduced to a novel adult while in the raphe nucleus the opposite was true. It was discovered that the majority of c-fos immunoreactivity in the PVN did not come from mesotocinergic neurones and there was no difference in the number of neurones positive for both c-fos and mesotocin between the two groups. To the author's knowledge, these findings describe for the first time the effects of previous cohabitation on the neuronal response of adult female quail to novel chicks and demonstrate that neurones in the PVN and nCPa are activated by the presence of chicks. Presence of chicks was also related in this study to lower activation of neurones in the raphe nucleus when compared to the presence of a novel adult. These results suggest that some affiliative behaviours, which may be precursors to maternal behaviour, might not be governed by mesotocin neurones in the absence of incubation, but other neurones in the PVN may be involved. The reason for the higher neuronal activation in the nCPa in the presence of chicks remains unclear as this area controls sexual behaviours and further studies are necessary to identify the nature of the neurones expressing c-fos in this nucleus under these conditions. The lower c-fos expression in the raphe nucleus in the presence of chicks compared to in the presence of an adult does not agree with the hypothesis that chicks are rewarding. It is possible that it is instead related to a lower stress-related activation of serotonergic neurones in this area induced by novel chicks as compared to novel adults. However,

unfortunately, a test for stress was not included in the present experiment so this should be the subject of further studies.

Explanatory note: *This experiment was originally designed to be performed in chickens. Due to husbandry issues at the Roslin Institute explained in detail in chapter 8, this was not possible, so the study was redesigned for quail.*

7.1 Introduction

7.1.1 Importance of stimuli from the young for maternal behaviour

A number of studies in both birds and mammals have demonstrated that maternal animals are highly responsive to cues coming from their offspring or from adoptees towards whom they are displaying maternal care. These stimuli can have both behavioural and physiological effects and can be crucial for the onset and maintenance of maternal behaviour (Thayananuphat et al., 2011; Richard-Yris et al., 1998; Edgar et al., 2011; Whitworth et al., 1984; Orpen et al., 1987; Pearson et al., 2011; Geissler et al., 2013; Mal'tsev et al., 1977; Smotherman et al., 1976).

7.1.1.1 Stimuli from the young and the onset of maternal care

While pregnancy in mammals and incubation in birds are periods during which the female brain changes in preparation for motherhood, stimuli from offspring are usually necessary to trigger the transition to maternal care and these stimuli can have very strong effects. In postpartum rat dams, exposure to pups for 30 min or longer caused a maternal response which was still present when dams were tested again through the introduction of pups 10 days later after being denied contact with pups (Orpen et al., 1987). Significantly, with shorter initial exposure time, post-partum dams were no more responsive than virgins during this test, underlining the importance of this contact with the young (Orpen et al., 1987). Similarly, a study in the chicken showed that tactile stimuli from chicks alone or possibly in combination with visual and auditory stimuli (but not visual and auditory stimuli alone) were necessary for the transition from incubation to rearing in hens which had been incubating eggs (Richard-Yris et al., 1998). In the same study, hens which were put in physical contact with young chicks displayed a rapid decrease in prolactin plasma concentrations, a slow increase in LH plasma concentrations, a gradual decrease in attachment to the nest and a rapid increase in signs of maternal care, including food calls and clucking (Richard-Yris et al., 1998). In the turkey, exposure of incubating hens to chicks also caused hens to rapidly display maternal behaviour (Thayananuphat et al., 2011).

7.1.1.2 Behavioural and physiological responses to cues from young

Apart from the initial onset of rearing behaviour, there are a number of physiological and behavioural responses which can be triggered in adults by

the presence of young. In a study in chickens, mother hens produced more food calls when they noticed their chicks were not feeding, which in turn caused chicks to feed (Wauters and Richard-Yris, 2002). Chicken mothers also showed increased maternal vocalisations when an aversive stimulus (a puff of air) was directed at their chicks, indicating an empathic response in the mother (Edgar et al, 2011). Similarly, when rat pups were exposed to noxious stimuli, this elicited a strong pituitary-adrenal response from the mother (Smotherman et al., 1976). In mice, ultrasound cues from pups with a specific length and frequency (long sounds at 50Hz) instigated in maternal animals a search for lost pups and triggered c-fos expression relevant to the news value of the sounds (adequate vs inadequate imitations of pup calls) in the POM, BnST and LS of both biological mothers and virgin mice which had been taking care of pups for 1 or 5 days, even though the behavioural response (pup retrieval) was significantly larger in mothers than in virgins (Geissler et al., 2013). In rat mothers, the absence of the pups or pain vocalisations from pups also induced a search and retrieval (Mal'tsev et al., 1977). Interestingly, in human females, breastfeeding increased the attentional sensitivity to infant distress as determined through attention and emotion recognition tests involving the scoring of human faces for what emotion they were showing (Pearson et al., 2011).

Along with these behaviours, some physiological changes can be caused by stimuli coming from the young. As mentioned previously, in the alloparental prairie voles the presence of pups caused increased heart rate in both sexes (Kenkel et al., 2015). In the chicken, in addition to increasing their maternal vocalisation hens also displayed increased heart rate in response to their chicks experiencing an aversive stimulus (Edgar et al., 2011). In the rat, the suckling stimulus from pups not only caused prolactin release but also helped maintain the responsiveness of the prolactin system to further suckling stimuli, as well as the behavioural patterns in the mother which allowed suckling (Whitworth et al., 1984). In the same study, mothers previously

exposed to the suckling stimulus began nursing their pups quicker than previously unexposed controls (Whitworth et al., 1984).

7.1.2 Induction of maternal behaviour in animals that have not undergone parturition or incubation

While it is possible to induce maternal behaviour in female animals which are not biological mothers, some difficulty is presented by the fact that animals which have not undergone parturition or incubation often have negative responses to young conspecifics. Non-maternal female chickens have been shown to display aggression towards chicks (Richard-Yris et al., 1983) and virgin rat dams showed aversion to pups although, interestingly, juvenile females found pups less aversive, interacted more with pups and became maternal quicker than adult virgin females (Olazábal et al., 2006).

However, sufficiently long contact with young eventually induces parental behaviour in many species. Virgin adult female mice showed maternal behaviour very readily after exposure to pups, indicating that the behaviour can be induced by contact independent of the hormonal environment or events at parturition in this species, even though maternal aggression could not be induced even in fully maternal virgin females in this study (Martín-Sánchez et al., 2015). In addition, female virgin mice presented with live day-old pups built larger nests and more nests classified as ‘maternal’ than virgins presented with no pups or dead pups, demonstrating that not only the physical presence but the behaviour of the pups is necessary to induce a behavioural change in the adult (Gandelman, 1973). Virgin female golden hamsters (Swanson et al., 1979) and virgin female rats (Jakubowski et al., 1980) could also be induced to become maternal through exposure to pups

and it was demonstrated in the rat that contact with the pups induced prolonged diestrus in maternal virgin females (Jakubowski et al., 1980).

Maternal behaviour can be achieved in avian species in a similar way. Broody behaviour was induced in Japanese quail by repeated 20-min exposures to 2-5-day old chicks (Ruscio et al., 2004). Maternal behaviour (coinciding with the cessation of egg-laying and decrease in sex steroid concentrations) was successfully induced by the introduction of young chicks in laying hens and hens which had been artificially stopped from laying (Richard-Yris et al., 1983) although the development of maternal responses was slower in non-incubating birds than in incubating hens (Richard-Yris et al., 1987).

7.1.3 Neuronal activation in the maternal brain

Increased expression of c-fos, an immediate early expression gene often used to identify neurones which have fired recently, has been observed in the brains of both birds and mammals during displays of maternal behaviour (Thayananuphat et al., 2011; Ruscio et al., 2004; Fleming et al., 1994; Numan and Numan, 1997; Lonstein et al., 1998; Numan et al., 1998; Da Costa et al., 1999; Katz et al., 1999). In maternal turkey hens exposed to chicks, c-fos expression was induced in a number of brain areas relevant to maternal behaviour including the PVN, POM, BnSTm, SON, lateral hypothalamus (LHy), inferior hypothalamus (IH), infundibular nucleus (IN) and lateral mamillary nucleus (ML) (Thayananuphat et al., 2011). Female quail which displayed maternal behaviour (crouching and covering chicks with wings) as the result of the introduction of chicks, had higher c-fos in the BnSTm than quail that did not respond maternally to the chicks under the same conditions (Ruscio et al., 2004). In sheep, maternal behaviour was

induced at birth together with the release of oxytocin, and c-fos in the PVN was elevated (Da Costa et al., 1999). Similarly, in prairie voles there was an induction of c-fos in the PVN, SON, LS, BnST, and MPOA during the first 24 hours postpartum (Katz et al., 1999). Rat females interacting maternally with pups also showed elevated c-fos expression in the MPOA (Fleming et al., 1994; Numan and Numan, 1997; Lonstein et al., 1998; Numan et al., 1998), the BnSTm (Numan and Numan, 1997; Lonstein et al., 1998; Numan et al., 1998) the LS, the nucleus accumbens and the lateral habenula (Lonstein et al., 1998).

7.1.4 Aim and hypothesis

In this study, the aim was to determine whether female quail habituated to chicks would respond differently to novel chicks than to a novel adult. Another aim was to attempt to establish which brain regions, previously associated with maternal behaviour in studies in mammals and birds, were responsible for the onset of rearing behaviour in the quail and whether the brain mesotocin system recently shown to be important for maternal care was involved in the response to chicks in this species. Rearing was separated from incubation through the use of female Japanese quail which rarely incubate in captivity but have been known to adopt chicks without displaying harmful aggressive behaviours. The design simulated cohabitation with younger siblings - a more natural situation than paradigms used previously - to induce a maternal response in precocial birds. The differential response to chicks vs adults was tested after a period of cohabitation and the c-fos expression induced by the presence of chicks was examined in areas of the social behaviour network in the brain.

Hypothesis:

Based on previous studies cited above, it was hypothesised that adult female quail which had cohabitated with chicks would spend more time in proximity to novel chicks compared to a novel adult individual. It was expected that the PVN, BnST, POM, LS, SON and raphe nucleus of these quail would become activated in the presence of chicks while activation in the nCPa decreased, as it was expected that sexual behaviours would be suppressed by the presence of the chicks (although sexual behaviour was not tested for in these experiments). It was also hypothesised, based on existing evidence for the involvement of mesotocin in maternal behaviour, that mesotocinergic neurones in the PVN specifically would become activated in the presence of chicks.

7.2 Materials and methods

7.2.1 Animals and housing

Adult female Japanese quail and young quail chicks, at least six days old at the start of the experiment, were obtained from a colony maintained at the Roslin Institute Poultry Unit and used in this study. The birds were kept in $2m^2$ floor pens supplied with heat lamps and appropriate drinkers and feeders for both the adults and the chicks. Food and water were provided *ad libitum*. Lights were turned on at 6am for a 14:10h light/dark cycle.

7.2.2 Experimental design

Sixteen adult female quail which had been housed in pairs for at least a week were introduced to 5 six-day-old chicks per pair and allowed to cohabitate with the chicks in their home pen for 6 days. On the night of the sixth day the chicks were removed and the next morning a new group of five six-day-old chicks were introduced into the pens of 4 of the pairs and a novel adult individual was introduced into the pens of the remaining four pairs (controls). The birds which received chicks and those receiving adults were housed in separate experimental rooms. The behaviour of the birds was recorded in the 90 minutes following the arrival of the new individuals, after which birds were euthanised with an overdose of anaesthetic (pentobarbital delivered through injection into the wing vein) and their brains were collected in pairs of one bird per experimental group to control for possible effects of the time of euthanasia.

7.2.3 Behavioural observation and analysis

The behaviour of naive adult female quail was observed without being quantified in the first half hour after they were introduced to chicks for the first time. For the 90-min neuronal activation experiment digital video cameras were installed outside of the pens to record the behaviour of all birds. These video recordings were subsequently analysed visually without the help of specialised software as this was not deemed necessary. The time spent in proximity to any number of chicks or to the novel adult individual was recorded for each experimental bird. Quail were considered to be ‘in proximity’ to chicks or to the novel adult when at least one chick or the novel adult were within a distance no greater than the size of one adult quail. No

displays of aggression towards the chicks were observed and there was no severe aggression towards the novel adults but in the instances when experimental females chased the novel adult around the pen in a display of aggression any proximity between the birds was disregarded as this was not considered an affiliative behaviour.

7.2.4 Brain collection and sectioning

The entire brain of each bird was dissected out and immediately fixed overnight in 4% paraformaldehyde in 1xPBS (diluted from stock 10x solution containing (1.4M NaCl, 0.027M KCl, 0.1M Na_2HPO_4, 0.018M KH_2PO_4) in 50ml tubes. The brains were then transferred to first 15% and then 30% sucrose in 1xPBS until they had sunk to the bottom of the tube. After this cryoprotection step was complete, the brains were dried carefully and frozen at -70°C until sectioning. Brains were sectioned coronally on a microtome at 50µm and sections were kept in cryoprotectant solution (containing 50% 2X PBS, 30% Ethylene glycol and 20% Glycerol, pH 5.5, stored at -20ºC until use).

7.2.5 Immunohistochemistry

7.2.5.1 Immunohistochemistry on free-floating sections for c-fos

In this procedure, the aim was to assess the level of activation of the nervous system by measuring the expression levels of an indirect marker. C-Fos is an

immediate early expression gene which is used in research as an indicator of recent neuronal activity as it is often expressed while a neuron fires action potentials.

As the goal was to perform double labelling (described further below) on the quail brains, thin fresh frozen sections could not be used. Therefore, pre-fixed free-floating sections 50µm in thickness were used instead. The use of thicker fixed sections also allows for the more detailed visualisation of neurons and fibres. All washes were completed in metal baskets in individual Petri dishes. A shaking platform was used to ensure effective washing or even spread of the reagents for all sections. Slides were given four 15-min washes in 1xPBS-T and one 5-minute wash in 1xPBS. Endogenous peroxidases were quenched with 0.3% H_2O_2 in 1xPBS for 20 min. Slides were washed three times for 10 min in 1xPBS-T. Washes done at 80 rpm on a shaking platform. Non-specific binding was blocked with blocking solution consisting of 10% NGS in PBS-T for 60 min on shaker at 80rpm. Sections were incubated overnight at 4°C in separate vials with 4ml antibody solution per vial in K-25 primary c-Fos antibody (Santa Cruz) diluted in blocking solution to a concentration of 1:10 000. After this incubation sections were washed three times for 10 min in 1xPBS-T. Secondary antibody solution was prepared (described in the Material and Methods section of Chapter 3). Sections were incubated in secondary antibody for 60 min on shaker at RT at 100 rpm in vials with 4ml solution per vial and then washed three times for 10 min in 1xPBS-T. ABC solution was prepared 30 min before use (described in the Materials and Methods section of Chapter 3). Sections were washed three times for 10 min in 1xPBS-T and once for 5 min in 0.1M sodium acetate buffer. Visualisation solution consisted of 0.08% ammonium chloride, 2.5% nickel ammonium sulphate and 1% DAB in 0.1M sodium acetate buffer with 0.03% H_2O_2 added just before use and mixed well. The addition of ammonium chloride and nickel ammonium sulphate intensifies signal in this procedure.

Nickel sulphate and ammonium chloride were dissolved in 0.2M sodium acetate buffer and DAB was dissolved in an equal volume of dH_2O before the two solutions were combined and H_2O_2 was added and sections were immersed in the solution. Reaction was halted after 10 min with 0.1M sodium acetate buffer for 5 min on shaker at 80rpm. Sections were washed five times for 5 min in PBS. If only labelling for c-fos, at this point sections were stored in 1xPBS at 4°C until mounting. Sections were then float mounted on gelatinised slides, left to air dry, then dehydrated through an alcohol series (70% and 95% ethanol and xylene) and coverslipped within two days of visualisation.

7.2.5.2 Double labelling for c-fos and mesotocin

Double labelling for c-fos and another protein of interest - in this case mesotocin – made it possible to determine whether mesotocinergic neurons became activated under the examined conditions. Continuing on from the c-fos IHC procedure described above, sections were washed twice for 10 min in PBS-T and twice for in PBS for 5 min. Endogenous peroxidases were quenched again as above and sections were washed twice for 10min in PBS-T. Sections were then incubated in blocking solution prepared as above for 1 hour and subsequently incubated in 1:1000 Oxytocin antibody (as used in Chapter 3) in vials at 4°C for 48 hours. After this incubation, sections were given three 10-min washes in 1xPBS-T and incubated in secondary antibody (Vectastain Elite Rabbit IgG ABC Kit) prepared as above for 60 min on shaker at 100rpm. Sections were given three 10-min washes in 1xPBS-T and then incubated for 60min on shaker at 100rpm in ABC solution prepared as above 30 min before use. After this incubation slides were washed three times for 10 min in 1xPBS-T and once for 5 min in 1xPBS. Cells were

visualised in 1% DAB in PBS with 0.03% H_2O_2 added and stirred just before use. Reaction was stopped by immersing sections in 1xPBS before they were washed five times for 5 min in 1xPBS. Sections were stored in 1xPBS at 4°C until mounting. Sections were dehydrated, mounted and coverslipped as described above within two days after visualisation.

7.2.6 Image analysis

The number of cells positive for c-fos or c-fos and mesotocin were counted under a light microscope in the region of interest (ROI) either per hemisection (LS, PVN, BnSTm, SON) or in the entire region when the border between the left and right hand side of the brain was difficult to determine (POM, nCPa, dorsal and median raphe nuclei, which were examined together and referred to as 'the raphe nucleus' for the purpose of this study). 10x, 20x or 40x magnification was used, sometimes alternating between the three, depending on how robust the signal was, or, in the case of double-labelled cells, how easy double labelling was to identify. Between 3 and 4 hemisections per bird were analysed for the PVN for both c-fos and double labelling, the SON, the LS and the BnSTm. Damaged sections were excluded and the average of all counts per bird was used for statistical analysis. 2 whole sections per bird were analysed and the average of the two counts was used for statistical analysis the POM and 1 whole section per bird was analysed in the raphe nucleus. For the nCPa, one whole section from the single-labelled c-fos immunohistochemistry and one whole section from the double-labelled c-fos and mesotocin immunohistochemistry was analysed for c-fos nuclei and the average of the two was used for statistical analysis.

7.2.7 Statistical analysis

SigmaPlot software was used in the performance of all statistical tests. All data was normally distributed. Student's t-test was used to test for differences between groups when the Brown-Forsyth equal variances test was passed, and Welch's test was used when the equal variances test failed. $P<0.05$ was considered statistically significant.

7.3 Results

7.3.1 Behaviour of naive quail presented with chicks

During the first 30 minutes after being presented with chicks for the first time, adult females exhibited fearful and aversive behaviour keeping a distance from the chicks and running away when a chick attempted to approach them. No aggressive behaviours towards the chicks were observed.

7.3.2 Behaviour of habituated quail presented with chicks

Photographs of experimental birds interacting with a novel adult or novel chicks can be seen on Figure 7.1A while figure 7.1B shows a graphical representation of the difference in time spent in proximity to chicks vs novel adult.

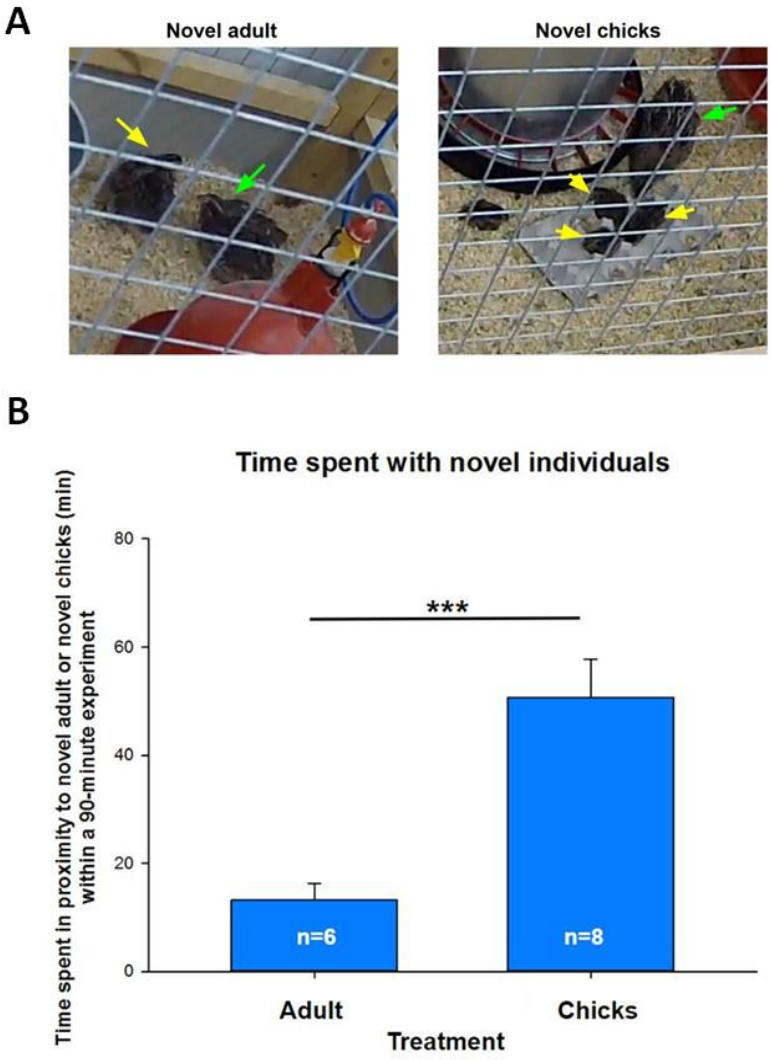

Figure 7.1 Interactions of adult female quail with a novel adult vs novel chicks.

A. Female quail interacting with a novel adult (left) and novel (chicks). Experimental birds are marked with green arrows. Novel birds introduced to the pen are marked with yellow arrows.

B. There was a significant difference between groups with experimental birds spending more time with novel chicks compared to novel adult (Student's t-test, $t_{(12)} = -4.955$, $p<0.001$. n=6-8. Data for two of the birds in the group introduced to a novel adult was not available due to a camera malfunction. Data are presented as the mean +SEM. *** denotes $p<0.001$).

Females which had been cohabitating with chicks for six days did not show any obvious fearful or aversive behaviours (running away when chicks approached, chasing chicks or pecking chicks) after the introduction of novel chicks. They approached the chicks on many occasions and allowed chicks to approach them, they were observed sitting, pecking and drinking in proximity to chicks. Although no visible signs of maternal behaviour (crouching posture, spreading wings and covering chicks) were observed, experimental females spent significantly more time in proximity to chicks than to a novel adult individual (Student's t-test, $t_{(12)}$=-4.955, $p < 0.001$).

7.3.3 Effect of chicks on c-fos immunoreactivity in the brain of adult female Japanese quail

A schematic representation of the density of the Fos nuclei observed in the hypothalamus is shown on Figure 7.2.

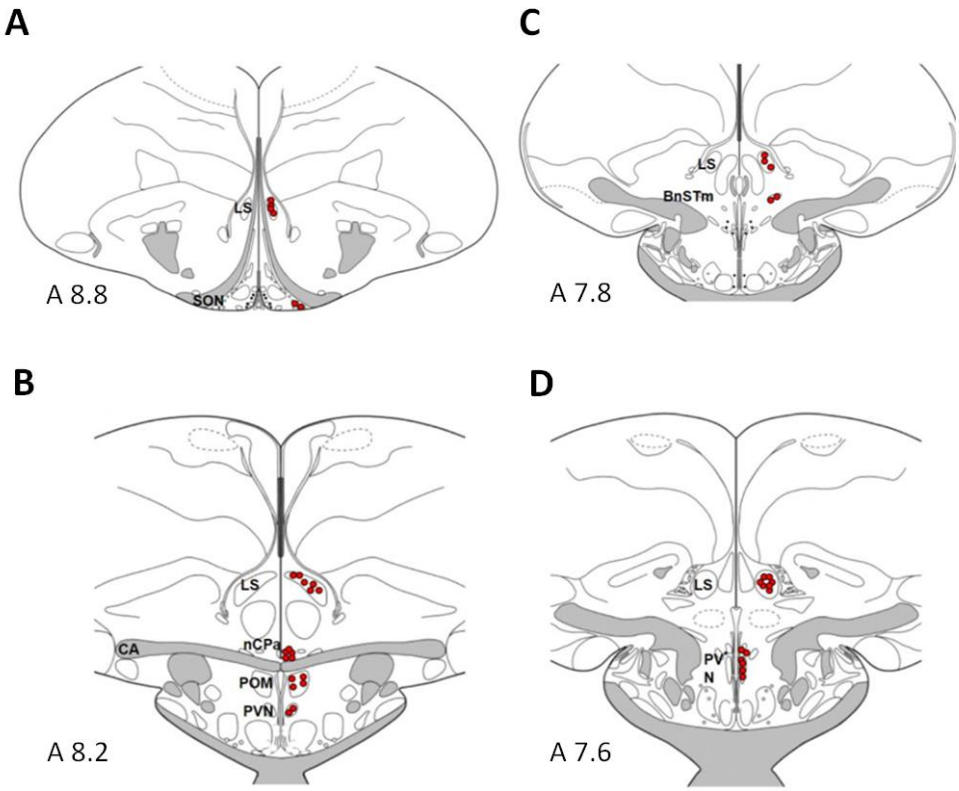

Figure 7.2 Fos nuclei in the hypothalamus of adult female quail.

Schematic diagrams of coronal sections illustrating the distribution of Fos nuclei (red dots) in the hypothalamus of adult female Japanese quail. Illustrations are adapted, with the given coordinates (lower left corner of each image), from the stereotaxic atlas of the brain of the chick (Kuenzel and Masson, 1988), where 'A' followed by a number signifies a brain plate the given number of millimetres anterior to a zero reference plane. Fos nuclei were densest in the lateral septum (LS) and the nucleus comissurae pallii (nCPa), relatively dense in the medial preoptic area (POM) and the paraventricular nucleus (PVN) and less dense and fainter in the supraoptic nucleus (SON) and the medial bed nucleus of the stria terminalis (BnSTm).

Out of the areas examined in this study, the largest number of nuclei positive for Fos and the strongest staining was observed in the nCPa and the LS. Labelled Fos nuclei were also relatively dense in the POM directly under the

CA, the raphe nucleus and occasionally in the PVN. In the BnSTm and SON c-fos nuclei were few and staining was relatively faint. Photomicrographs showing the staining in the examined areas, together with graphical representation of the results can be seen on figures 7.3 (POM), 7.4 (SON), 7.5 (LS), 7.6 (BnSTm), 7.7 (nCPa), 7.8 (PVN) and 7.9 (raphe nucleus). No difference between groups was found in the POM (Student's t-test, $t_{(11)} = -0.267$, $p = 0.794$), SON (Student's t-test, $t_{(10)} = -0.554$ $p = 0.591$), LS (Welch's test, $t_{(11.476)} = 0.343$, $p = 0.738$) or BnSTm (Welch's test, $t_{(5.276)} = 0.202$ $p = 0.848$) but there was a difference in the nCPa, PVN and the raphe nucleus. Fos-immunoreactive nuclei were more numerous in quail presented with chicks in the PVN (Student's t-test, $t_{(9)} = -2.339$, $p = 0.044$) and nCPa (Student's t-test, $t_{(11)} = -2.204$, $p = 0.050$) compared to quail presented with a novel adult. The opposite was the case in the raphe nucleus (Student's t-test, $t_{(8)} = 3.105$, $p = 0.015$)

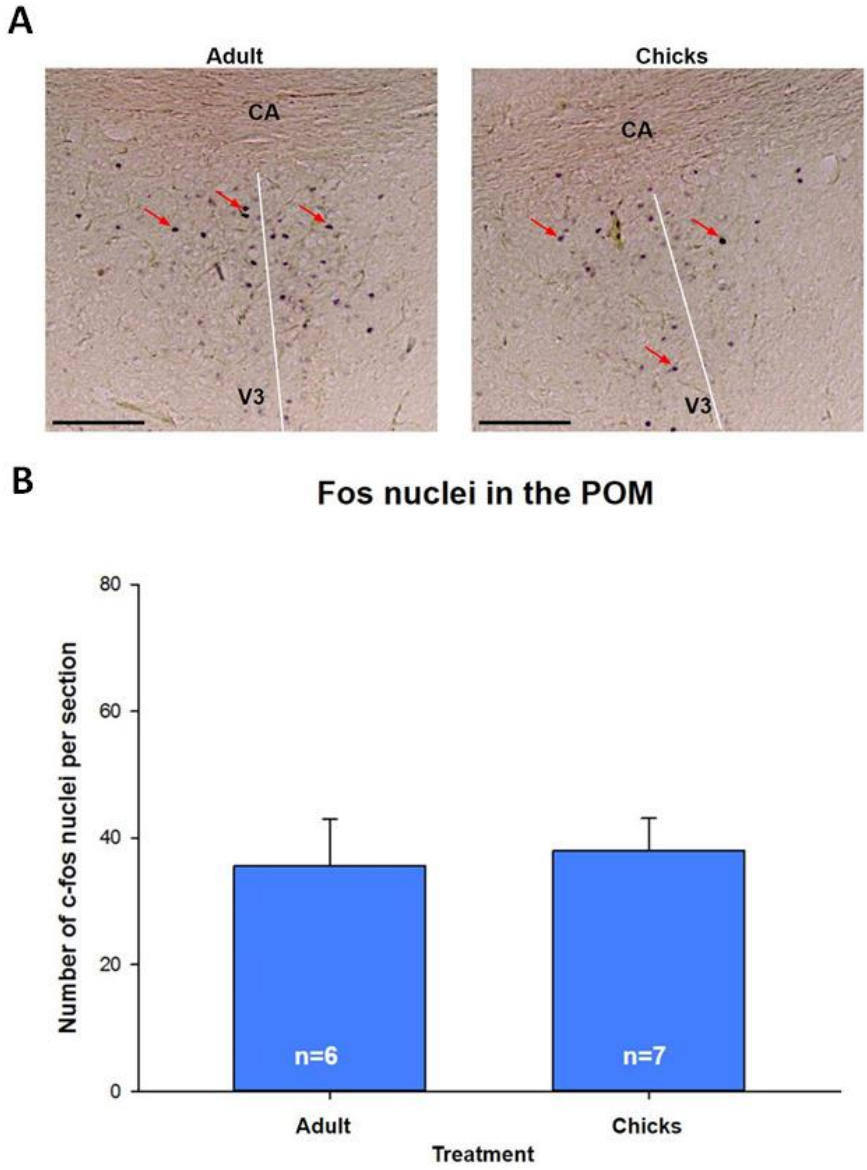

Figure 7.3 C-fos immunoreactivity in the POM induced by the presence of a novel adult or chicks.

A. Photomicrographs show c-fos positive nuclei (black dots) in the POM of experimental birds introduced to either a novel adult or five novel chicks. The position of the third ventricle is shown (V3 and white line). Arrows denote examples of labelled nuclei. Scale bar = 100µm.

B. There was no significant difference between groups (Student's t-test, $t_{(11)}$=-0.267, p = 0.794) n=6-7. (Individuals were excluded from analysis where sections containing the region of interest were lost during cryosectioning. As the brains were small and sections were thick the loss of a small number of sections had a large impact.) Data are presented as the mean +SEM.

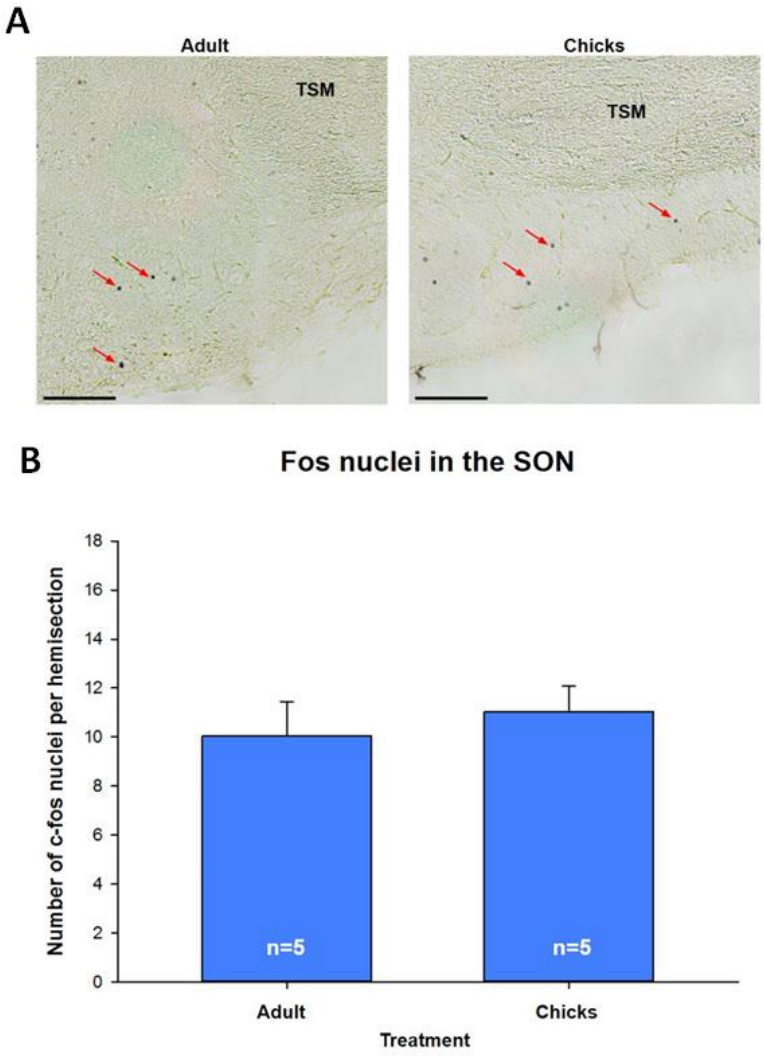

Figure 7.4 C-fos immunoreactivity in the SON induced by the presence of a novel adult or chicks.

A. Photomicrographs show c-fos positive nuclei (black dots) in the SON of experimental birds introduced to either a novel adult or five novel chicks. The position of the ceptomesencephalic tract (TSM) is shown. Arrows denote examples of labelled nuclei. Scale bar = 100µm.

B. There was no significant difference between groups (Student's t-test, $t_{(10)} = -0.554$, $p=0.591$). n=5. (Individuals were excluded from analysis where sections containing the region of interest were lost during cryosectioning. As the brains were small and sections were thick the loss of a small number of sections had a large impact.) Data are presented as the mean +SEM.

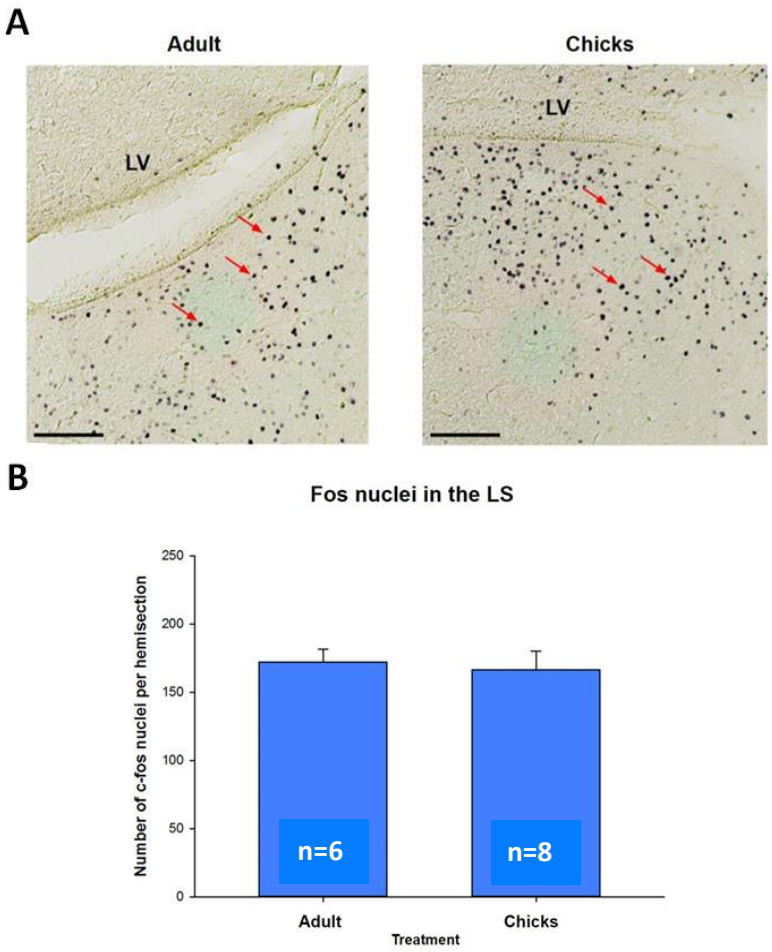

Figure 7.5 C-fos immunoreactivity in the LS induced by the presence of a novel adult or chicks.

A. Photomicrographs show c-fos positive nuclei (black dots) in the LS of experimental birds introduced to either a novel adult or five novel chicks. The position of the lateral ventricle (LV) is shown. Arrows denote examples of labelled nuclei. Scale bar = 100µm.

B. There was no significant difference between groups (Welch's test, $t_{(11.476)}$=0.343, p= 0.738). n=6-8. (Individuals were excluded from analysis where sections containing the region of interest were lost during cryosectioning. As the brains were small and sections were thick the loss of a small number of sections had a large impact.) Data are presented as the mean +SEM.

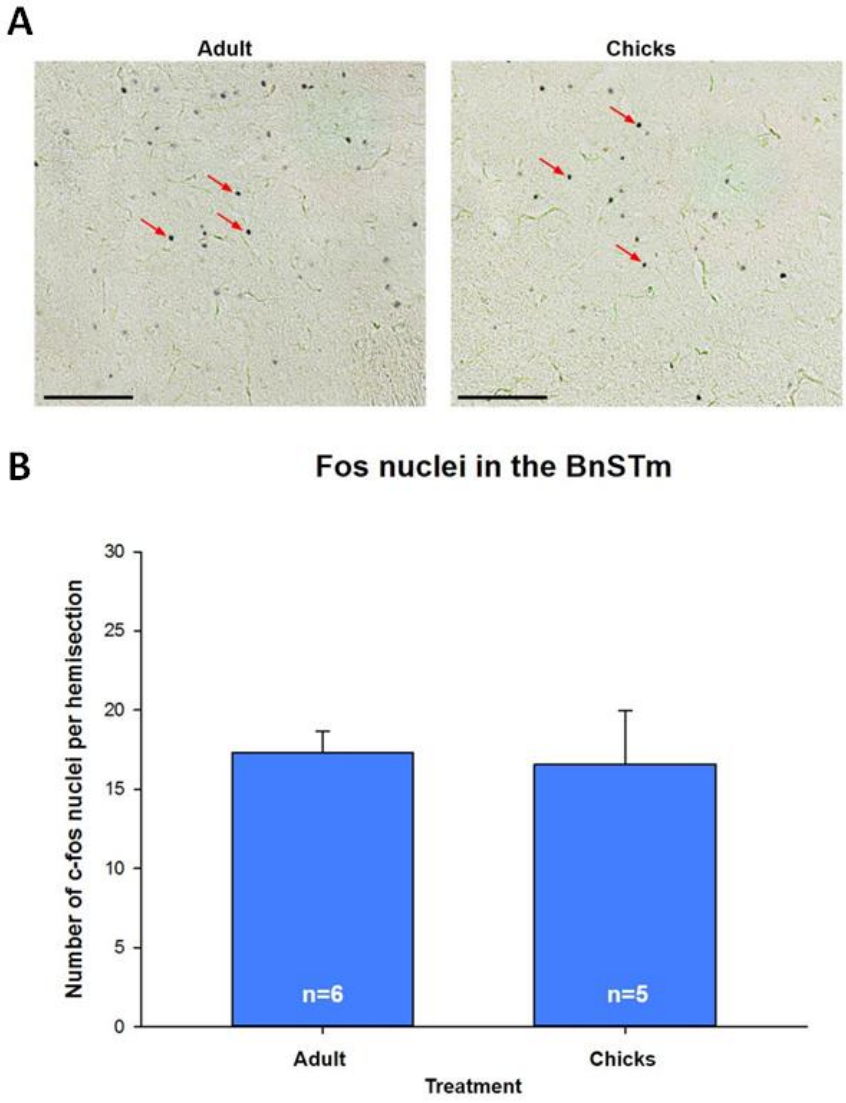

Figure 7.6 C-fos immunoreactivity in the BnSTm induced by the presence of a novel adult or chicks.
A. Photomicrographs show c-fos positive nuclei (black dots) in the BnSTm of experimental birds introduced to either a novel adult or five novel chicks. Arrows denote examples of labelled nuclei. Refer to Figure 7.2C for position where nuclei were counted. Scale bar = 100µm.
B. There was no significant difference between groups (Welch's test, $t_{(5.276)}$=0.202, p=0.848). n=5-6. (Individuals were excluded from analysis where sections containing the region of interest were lost during cryosectioning. As the brains were small and sections were thick the loss of a small number of sections had a large impact.) Data are presented as the mean +SEM.

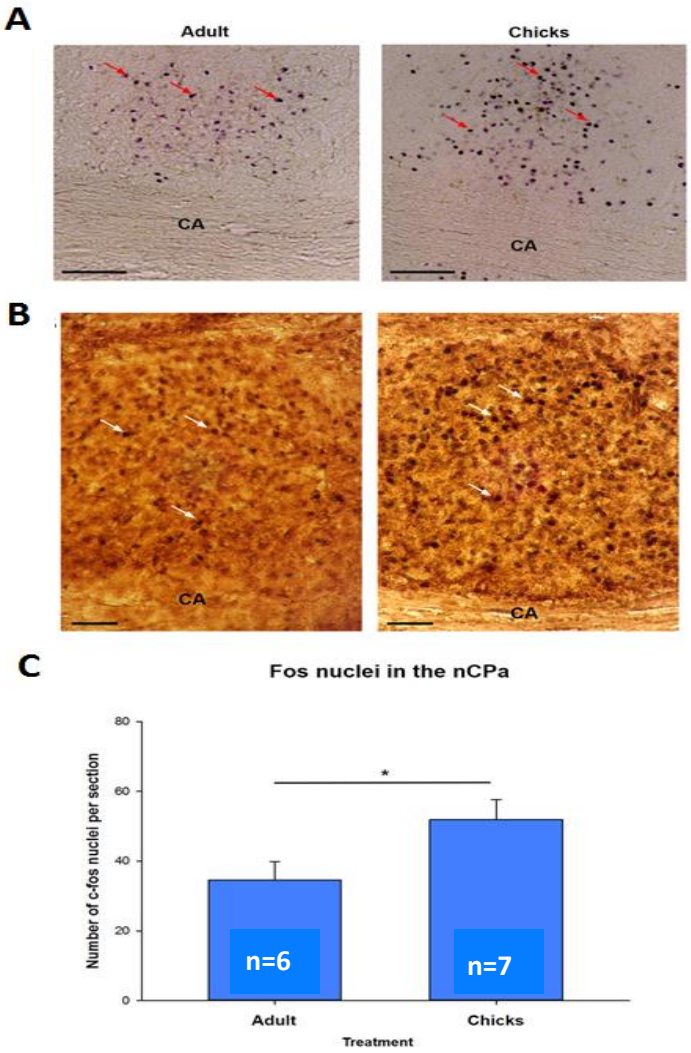

Figure 7.7 C-fos immunoreactivity in the nCPa of adult female quail introduced to a novel adult or chicks.
Photomicrographs show single labelling for c-fos (A) and c-fos nuclei after double labelling for mesotocin (however, no mesotocin cells are present on the images) (B). Images show c-fos positive nuclei (black dots) in the nCPa of experimental birds introduced to either a novel adult or five chicks. In B double-labelling for mesotocin increases background even though no double-labelled cells are present in the image as no mesotocin cells were found in that area and only c-fos was quantified. Arrows denote examples of labelled nuclei. Scale bar = 100µm for A and 50µm for B.
C. There was a significant difference between groups with experimental birds who received chicks showing a higher number of c-fos nuclei in the nCPa compared to those who received a novel adult (Student's t-test, $t_{(11)} = -2.204$, $p = 0.0498$). n=6-7. (Individuals were excluded from analysis where sections containing the region of interest were lost during cryosectioning. As the brains were small and sections were thick the loss of a small number of sections had a large impact.) Data are presented as the mean +SEM. * denotes $p<0.05$.

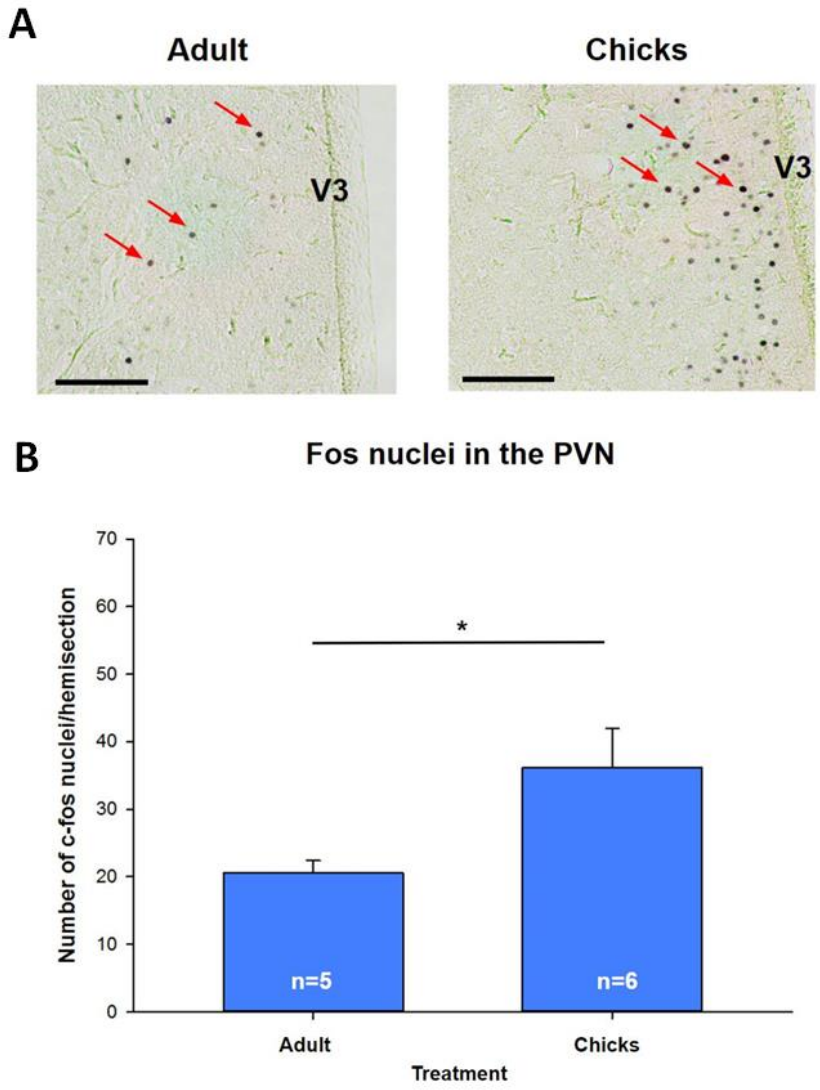

Figure 7.8 C-fos immunoreactivity in the PVN of adult female quail introduced to a novel adult or chicks.
A. Photomicrographs show c-fos positive nuclei (black dots) in the PVN of experimental birds introduced to either a novel adult or five novel chicks. The position of the third ventricle (V3) is shown. Arrows denote examples of labelled nuclei. Scale bar = 100µm.
B. There was a significant difference between groups with experimental birds who received chicks showing a higher number of c-fos nuclei in the PVN compared to those who received a novel adult (Student's t-test, $t_{(9)}$= -2.339, p=0.0441). n=5-6. (Individuals were excluded from analysis where sections containing the region of interest were lost during cryosectioning. As the brains were small and sections were thick the loss of a small number of sections had a large impact.) Data are presented as the mean +SEM. * denotes $p<0.05$.

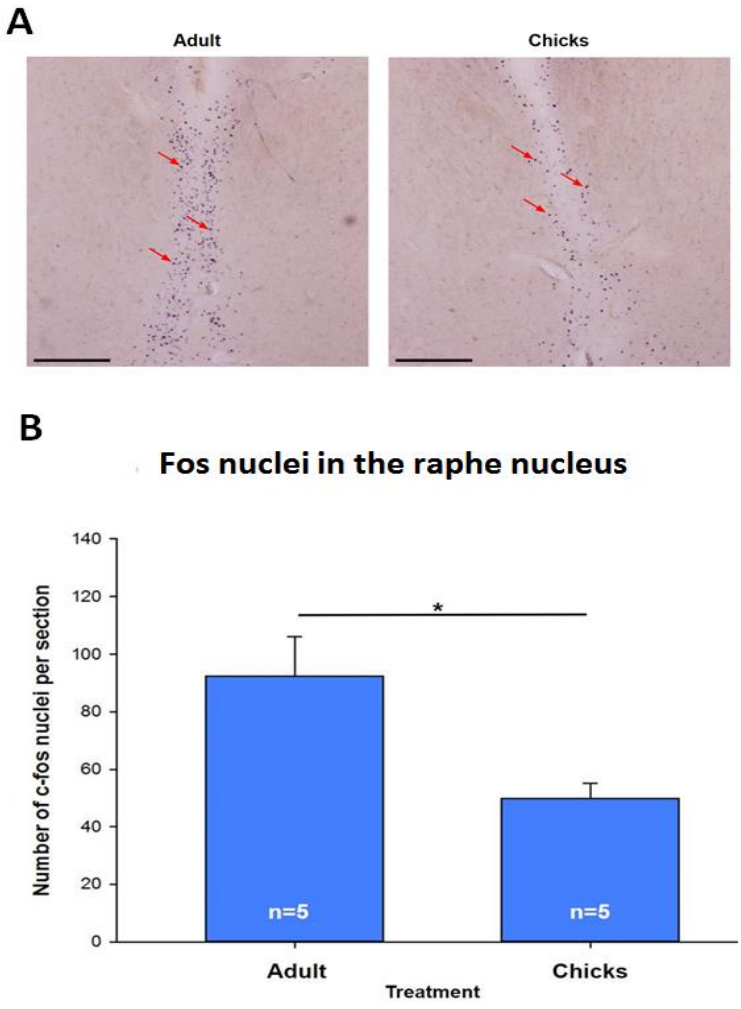

Figure 7.9 C-fos immunoreactivity in the raphe nucleus induced by the presence of a novel adult or chicks.
A. Photomicrographs show c-fos positive nuclei (black dots) in the raphe nucleus of experimental birds introduced to either a novel adult or five novel chicks. Arrows denote examples of labelled nuclei. Scale bar = 200µm.
B. There was a significant difference between groups with experimental birds who received chicks showing a lower number of c-fos nuclei in the raphe nucleus compared to those who received a novel adult (Student's t-test, $t_{(8)}$ = 3.105, p = 0.0146). n=5. (Individuals were excluded from analysis where sections containing the region of interest were lost during cryosectioning. As the brains were small and sections were thick the loss of a small number of sections had a large impact.) Data are presented as the mean +SEM. * denotes p<0.05.

7.3.4 C-fos expression from mesotocin neurones in the paraventricular nucleus

No difference was found in the number of neurones in the PVN co-expressing mesotocin and c-fos and very few double-labelled cells were identified (Student's t-test, $t_{(11)} = 0.925$, $p = 0.375$). An example of a double-labelled cell along with graphical representation of results is shown on Figure 7.10.

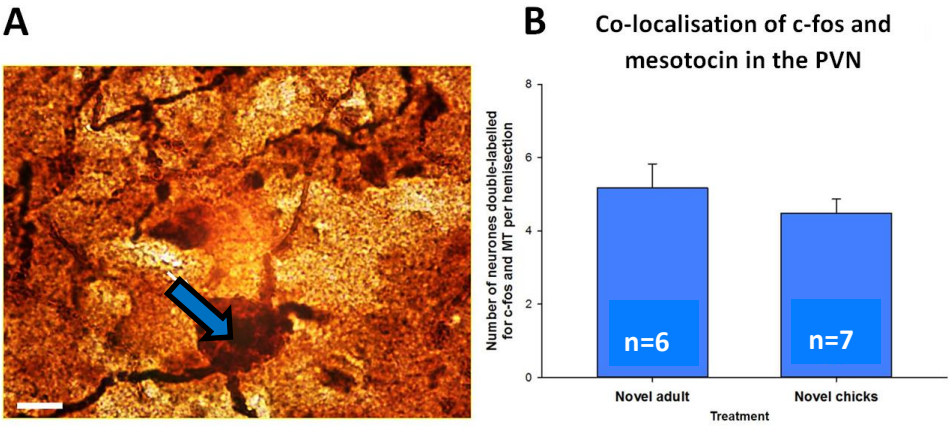

Figure 7.10 Colocalisation of c-fos and mesotocin in the PVN.
A. Light-field photomicrograph showing an example of a cell double-labelled for c-fos and mesotocin in the PVN. An arrow denotes the double-labelled cell. The c-fos nucleus (black chromogen) can be seen inside the cytoplasm of a mesotocin-immunoreactive neurone. Scale bar = 20µm.
B. There was no significant difference between groups (Student's t-test, $t_{(11)} = 0.925$, $p=0.375$). (Individuals were excluded from analysis where sections containing the region of interest were lost during cryosectioning. As the brains were small and sections were thick the loss of a small number of sections had a large impact.) n=6-7. Data are presented as the mean +SEM.

7.4 Discussion

7.4.1 Effects of chicks on female behaviour

Cohabitation with chicks for six days in this study had a clear effect on the subsequent response to novel chicks. All adult quail in the experiment were fearful and actively avoided chicks when first introduced to them, running away when a chick approached, although no signs of aggression were noted. This behaviour was the opposite of what was observed when quail were introduced for the first time to a novel adult, as in that case the residents of the pen did not appear fearful and, in fact, occasionally displayed signs of aggression by chasing the new individual around the pen. These observations are in agreement with studies in other species, including rats and chickens, in which non-maternal females have been shown to display negative reactions to young of the same species (Richard-Yris et al., 1983; Olazábal et al., 2006). However, by the sixth day of sharing a pen, quail were no longer running away from chicks and appeared to comfortably share feeders and drinkers and sit together, indicating that the presence of incubation or a hatching event is not necessary to abolish the negative response to chicks in this species. Females exhibited the same behaviour towards the group of novel chicks which was introduced during the behavioural test, confirming that these positive behavioural responses were not connected to the familiarity of the individual chicks but rather represented a change in the response to chicks in general. This finding once again agrees with previous studies as cohabitation with young has been demonstrated to eventually induce a positive response in adult females, usually ultimately leading to maternal behaviour, and this can have long-term effects which can be observed regardless of the familiarity of the young with which the adult is

in contact (Richard-Yris et al., 1983; Olazábal et al., 2006; Martín-Sánchez et al., 2015; Gandelman, 1973; Swanson et al., 1979; Jakubowski et al., 1980; Ruscio et al., 2004; Richard-Yris et al., 1987). Females spent significantly more time in proximity to the novel chicks compared to the novel adult which indicates that the affiliative behaviour observed was specific to chicks and not the result of a general increase in sociality after sharing a pen with more individuals regardless of age. The chicks used in this study were 6 days old when they were first introduced to the adults and were released to roam free in the pen in contrast to previous studies in which day-old chicks were used and they were placed under hens during the night in a confined space (Richard-Yris et al., 1983; Ruscio et al., 2004; Richard-Yris et al., 1987). This, together with the relatively short period of cohabitation (six days) is the most likely reason why no actual maternal behaviour was observed in this experiment. In order to distinguish between these three possible factors - age of chicks, duration of induction and physical contact - additional studies can be conducted varying the age of the chicks, the duration of the induction period and the confinement area in order to facilitate more physical contact between the adults and chicks. It has been previously demonstrated that physical contact is necessary for the development of maternal behaviour while visual and auditory cues are not enough (Richard-Yris et al., 1998).

7.4.2 Effects of chicks on neuronal activation in the paraventricular nucleus and mesotocin

This study found a significant difference in c-fos immunoreactivity in the PVN between the group introduced to chicks and the group introduced to adults. However, the majority of Fos-positive nuclei did not seem to be co-localised with mesotocin-immunoreactive neurones so the identity of the cells

responsible for this difference is unclear. The PVN also contains an abundance of vasotocinergic cells but, as discussed in Chapter 4 of this thesis, vasotocin in the PVN was not found to be involved in the care of chicks. In addition, the distribution of vasotocin neurones in this area closely follows that of mesotocin neurones. Both vasotocin and GnIH in the PVN have been shown to be elevated by stress (Cecchi et al., 2002; Nagarajan et al., 2014; Selvam et al., 2013) but the females studied in this experiment exhibited no obvious signs to suggest that encounters with novel chicks after the habituation were more stressful than encounters with a novel adult. Unfortunately, as corticosterone concentrations were not measured, there was no quantifiable way of measuring stress to back up these observations. The primary role of GnIH is as an inhibitor of reproduction (Bentley et al., 2006; Johnson et al., 2007) and it is known that sexual behaviours decrease prior to the onset of maternal behaviour (Hall et al., 1986; Zadworny et al., 1988). It is therefore possible that GnIH neurones in the PVN become activated in the presence of chicks in order to begin the process of downregulating sexual activity in preparation for maternal care. Further studies with the same experimental design and involving double labelling for c-fos and vasotocin and GnIH respectively are necessary to determine whether these types of neurones become activated under the present conditions. Behavioural studies to test for sexual behaviour after cohabitation with chicks would help determine whether their presence is capable of triggering the downregulation of reproduction even in the absence of incubation. Finally, it is still possible that the activation of mesotocin neurones contributed for the difference in c-fos, but the resolution of our procedure was not high enough to detect a difference after double labelling. While a difference was observed between groups, c-fos immunoreactivity in the PVN was not overly abundant in either group and it is possible that experimental conditions in this study did not provide a strong enough stimulus to produce denser and stronger signal which may have allowed us to identify more

double-labelled cells and detect a more subtle difference. In addition, mesotocin-immunoreactive neurones near the third ventricle were very dense and often overlapped when observed under the microscope, possibly obscuring fos-positive nuclei and sometimes making it difficult to determine whether there was true co-localisation or not. As only cells which were clearly positive for both proteins were included in the analysis, this could have led to some positive cells being left out because they could not be clearly identified. Attempting to use thinner sections or varying antibody concentrations to fine-tune the strength of the staining may help in achieving higher resolution. Unfortunately, with the numbers of birds available for testing and the number of sections obtained per bird, this was not possible within the scope of this study.

7.4.3 Effects of chicks on neuronal activation in the nucleus of the commissurae pallii

As the nCPa mainly contains GnRH neurones (Fraley and Kuenzel, 1993; Kang et al., 2006; Thayananuphat et al., 2007; Sartsoongnoen et al., 2012; Chaiyachet et al., 2013a) and is involved in the control of sexual behaviours, it is surprising that increased neuronal activation was observed in this area in the presence of chicks. There is, at present, no explanation for the possible activation of GnRH neurones by interaction with chicks. While some GnIH cells were found in this area in a recent study in the chicken (Zhang et al., 2017), they were not abundant and the Fos-immunoreactive nuclei in this area were quite abundant in most birds so it seems unlikely that it is GnIH neurones that are becoming activated in the nCPa in the presence of chicks, as it was suggested may be happening in the PVN. However, it is impossible

to know the identity of these neurones without further studies with double labelling for both types of cells.

7.4.4 Effects of chicks on neuronal activation in the raphe nucleus

A decrease in c-fos immunoreactivity was observed in the raphe nucleus of adult females interacting with novel chicks when compared to those interacting with novel adults, although since only one section could be examined per bird, these results are being interpreted with caution. The raphe nucleus contains mainly serotonergic cells and, as young have been previously shown to be rewarding to the mother (Ferris et al., 2005; Kramer et al., 2005) and the raphe has also been shown to be involved in the control of juvenile 'babysitting' in rats (Harding and Lonstein, 2016), higher activation would have been expected in these cells in the presence of chicks. However, the opposite was true in this case. A possible explanation concerns the involvement of serotonergic neurones in this brain area in the stress response. As serotonin expression is elevated with stress, it is possible that after habituation, chicks present a less stressful stimulus to adult females compared to a novel adult. In order to determine if this is true, behavioural tests and measurements of plasma cortisol could be performed in combination with this experimental design.

Conclusion

This study analysed the effects of exposing adult female Japanese quail to 6-day-old chicks, after a 6-day-long habituation to chicks, on the behaviour and

neuronal activation in the brains of those adult females. The examined areas included the PVN, POM, BnSTm, LS, SON, nCPa and the raphe nucleus. The data presented in this chapter show that after habituation, adult individuals of this species show stronger affiliation to novel chicks than to novel adult conspecifiers. This behaviour does not appear to be controlled by mesotocin but is accompanied by the activation of unknown cells in the PVN and nCPa and a reduction of neuronal activity in the raphe nucleus. To the author's knowledge, this is the first study in a precocial bird species which attempts to mimic natural cohabitation with younger siblings and examine the neuroendocrine and behavioural precursors of maternal behaviour induced in non-mothers. In view of previous data on the localisation of GnIH neurones in the PVN of quail and their involvement in the downregulation of sexual behaviours, these findings suggest the possibility that in females which have had previous experience with young, the presence of chicks may induce a downregulation of the reproductive system by GnIH neurones in the brain as a precursor to the development of maternal behaviour. In addition, it can be speculated that in habituated birds, novel chicks may present a less stressful stimulus than a novel adult, thus not inducing an activation of serotonin neurones in the raphe nucleus, while such activation has been previously shown to be caused by stress. These findings contribute to the understanding of the environmental, social and hormonal factors involved in the control of maternal behaviour in precocial birds and their differential response to young and adult conspecifiers. They also provide opportunity for further studies on the possible involvement of mesotocin, vasotocin, GnIH, GnRH and serotonin in these processes and the effects of chick age on the response of adult females.

Chapter 8 General Discussion

8.1 Summary and discussion of results

The aim of this thesis was to address a gap in scientific knowledge which exists in the area of avian maternal behaviour. Even though many studies in mammals have focused on motherhood and the mechanisms which control it, the literature in birds is very limited. The roles in maternal care of the avian orthologues of the known mammalian neuropeptides oxytocin and vasopressin, known to promote this behaviour, have only been examined in a small number of studies. In addition, a number of other compounds such as gonadal steroid hormones, GnIH and monoamine neurotransmitters have shown some promise as direct or indirect regulators of maternal care but scientific data on their involvement in birds are also limited. In the case of GnIH, which was first discovered in Japanese quail relatively recently, studies on its effects on maternal care are equally sparse in both mammals and birds.

This project attempted to provide a broad, comprehensive look at the neuroendocrine regulation of avian maternal behaviour. It looked at the changes in the brain of a number of interconnected hormones throughout the reproductive cycle from egg-laying through to chick-rearing and examined some of the relationships between them.

Sexual behaviour needs to be inhibited in order for maternal behaviour to take place, and gonadal sex steroids have been known to affect nonapeptides in the brains of birds and mammals. Therefore, the aim was first to broaden our understanding of the effects of gonadal steroids on nonapeptides in the brain of the chicken. Acute injections of testosterone, estradiol or progesterone after a period of priming with the synthetic estrogen

DES were delivered to juvenile hens. The mRNA expression of vasotocin in the PVN and BnSTl was measured, along with the mRNA expression in the same brain areas, immunoreactivity in the PVN and receptor binding in the LS for mesotocin. No differences were found between groups in receptor binding in the lateral septum. However, mesotocin mRNA expression was found to be significantly higher in both the PVN and BnSTl of birds treated with testosterone while, surprisingly, mesotocin immunoreactivity was lower in the PVN with this treatment. This could have been due to mesotocin being quickly transported out of cells. Vasotocin mRNA expression in the BnSTl was found to be significantly higher both in birds treated with testosterone and in those treated with estradiol.

As testosterone had an effect on both nonapeptides examined, and as its concentration is known from previous studies to be under the control of GnIH (another peptide of interest in this project), a measurement of plasma testosterone was included in further experiments, in order to monitor the possible effects of this hormone on results for other peptides. The ELISA assay for testosterone throughout the reproductive cycle of the hen showed - in agreement with previous studies - that plasma testosterone decreased (and it could not be detected by the assay) after the onset of incubation and it was still undetectable in hens on the first day of rearing. Thus, no effects of testosterone on mesotocin and vasotocin during incubation and rearing were expected.

Mesotocin and vasotocin mRNA expression was quantified in the brain of the domestic hen in several brain areas known to be important for maternal behaviour including the PVN, POM, SON, BnSTm and also in the BnSTl which had not previously been implicated in maternal care. Brains were collected at four reproductive stages - egg-laying, the onset of incubation (day 3 of sitting on the eggs), 14 days into incubation (out of 21 in the chicken) and the first day of rearing chicks. An increase in mesotocin mRNA

expression in the PVN was observed between egg-laying and chick-rearing and a decrease in both mesotocin and vasotocin mRNA expression was found in the BnSTl with the onset of incubation. In the case of mesotocin, mRNA expression had recovered to levels comparable to those in laying birds by the first day of rearing in the segment of the nucleus situated under the lateral ventricle (BnSTl2) but not the segment right next to the ventricle (BnSTl1). mRNA expression had also not recovered by the first day of rearing for vasotocin anywhere in the BnSTl.

Using the same experimental brains as above, the GnIH mRNA expression was also measured in the PVN and LHy, along with GnIH immunoreactivity in the PVN, at the aforementioned reproductive stages. Although there was no detectable change in the number of mRNA-expressing neurones throughout the reproductive cycle in either area, there was a significant increase in the number of GnIH-immunoreactive neurones in the PVN at the 14th day of incubation. This was no longer present by the first day of rearing.

In the pituitary glands of the same birds, dopamine receptor D2 and prolactin mRNA expression was measured at the same time points in order to investigate the relationship between prolactin and the dopaminergic system in the chicken. Prolactin is a known regulator of incubation, elevated in the plasma of incubating birds. Dopamine, through its D2 receptor, has been shown in previous studies to inhibit prolactin secretion. However, no difference was found between groups in the pituitary mRNA expression for either prolactin or D2.

Similarly, even though some monoamines have been shown to promote maternal care in mammals, there was no detectable difference in the concentration of dopamine or its precursor DOPAC, 5-HT or its precursors tryptophan or hydroxyanthranilic acid, adrenaline or noradrenaline in the raphe nucleus of the same hens.

Finally, the effect of interactions with young chicks on the activity of the nervous system was examined in a pecocial bird species. Unfortunately, due to husbandry issues at the Roslin Institute Poultry Unit, this experiment could not be conducted in chickens, as originally planned. Therefore, Japanese quail were used instead. Naive quail were habituated to chicks for 6 days in order to eliminate their initial fearful response to young conspecifics. After this habituation, they were presented with either new chicks or a novel adult individual for at least 90 minutes - enough time for the indicator of neuronal activity c-fos to accumulate in the brain. Nuclei positive for c-fos were then quantified in most of the previously mentioned brain areas including the PVN, POM, LS, SON, BnSTm and the raphe nucleus, as well as the nCPa which controls sexual behaviours. Higher c-fos immunoreactivity was observed in the group presented with chicks in the PVN and nCPa and lower c-fos immunoreactivity was observed in this group in the raphe nucleus. Brain sections were then double-labelled for c-fos and mesotocin to determine whether mesotocin neurones in the PVN were the ones becoming activated. However, no difference in the number of double-labelled cells was found between groups. The implications of these findings are discussed below.

8.2 The effect of gonadal steroids on nonapeptide systems in the brain

To the author's knowledge, this is the first study on the effects of gonadal steroids on vasotocin mRNA expression in the BnSTl of birds. The changes in vasotocin mRNA expression observed after acute sex steroid treatment in juvenile female chicks may be related to changes in the stress response as both the BnSTl and vasotocin (Cecchi et al., 2002; Nagarajan et al., 2014; Selvam et al., 2013) as well as sex steroids (Critchlow et al., 1963; Dickens and Bentley, 2014; Handa et al., 2011; Klukowski et al., 1997; Larkin et al.,

2010; Nagra et al., 1965; Seale et al., 2005a; Seale et al., 2005b; Zysling et al., 2006) have been shown to be involved in stress.

No elevation of vasotocin mRNA expression in the PVN was observed in this experiment even though a previous study in quail found that both testosterone and estradiol had such an effect (Aste et al., 2013). This was likely due to the fact that only a single steroid injection was used in contrast to the study in quail in which birds were injected on 16 consecutive days.

This study's findings that both testosterone and estradiol promoted vasotocin mRNA expression in the BnSTl are consistent with the hypothesis that testosterone acts on brain vasotocin neuornes through its estradiol metabolite after aromatisation by the enzyme aromatase. Aromatase-immunoreactive cells have been found to be co-localised with vasotocin neurones in birds (Balthazart et al., 1997a; Miller et al., 1992). This hypothesis is also supported by data previous study which showed that estrogen but not androgen receptors were found in the hypothalamus of birds (Voigt et al., 2009).

Prior to this project, the effects of sex steroids on mesotocin in birds were not known. It has been demonstrated in this thesis for the first time that a single injection of testosterone after a period of priming with DES can increase mesotocin mRNA expression in both the PVN and BnSTl of juvenile female chickens. Unlike with vasotocin where estradiol had an effect similar to testosterone, estradiol did not affect mesotocin mRNA expression in either area in this study. This suggests that testosterone's effects on mesotocin in this case were direct and not through its estrogenic metabolite. As mentioned above, androgen receptors have not been documented in either of the examined areas in birds, so it is possible that testosterone affects neuropeptide neurones through their afferents. It is also possible that androngen receptors are present in this area in the chicken, even though they do not seem to be present in the quail.

Mesotocin immunoreactivity in the PVN was lower in the testosterone-treated group, compared to control, even though mRNA expression was higher. This was somewhat surprising but high rates of mesotocin release in the PVN could be responsible for the discrepancy.

Receptor binding for the mesotocin receptor in the LS also showed no difference between treatments. Previous work showed that testosterone could increase nonapeptide innervation in the LS without necessarily also causing an increase in receptor binding (Plumari et al., 2004). Therefore, even though a significant effect of sex steroids on mesotocin receptor binding might have been expected in this area, the lack of it is not a precedent.

8.3 Changes in nonapeptide mRNA expression in the brain of the domestic hen throughout the reproductive cycle

In agreement with previous studies in the chicken and turkey (Chokchaloemwong et al., 2013; Thayananuphat et al., 2011), the results from this project suggest that the mesotocin system in the PVN is important for chick-rearing in birds. A significant difference was found between laying and rearing birds in mesotocin mRNA expression in this area. Surprisingly, the differences in expression between laying and the 14th day of incubation were not significant and no difference between the reproductive stages was found in the POM. A difference was expected, since a previous study in the Thai hen found differences in the number of mesotocin-immunoreactive neurones in the PVN and in the POM between laying and rearing hens as early as day 10 of incubation (Chokchaloemwong et al., 2013). The lack of difference in the results presented in this thesis could be due to differences in the rate of transcription and translation or natural differences in the strains of chicken. In addition, the hens in the aforementioned study had been rearing

chicks for 14 days while in the experiments in this project, the brains were collected on the first day of rearing. This may indicate that the POM is more important at a later stage and not at the transition from incubation to rearing.

The BnST is known to have a function in sexual and social behaviours in both mammals and birds and its lateral sector is involved in aggression and stress (Nagarajan et al., 2014) but the BnSTl had not been studied in relation to incubation and rearing before. The results presented in this thesis showed that mesotocin mRNA expression was significantly lower both at the onset of incubation and on the 14th day of incubation compared to expression in laying and rearing birds. In the sector of the BnSTl located under the lateral ventricle (but not in the sector directly next to the ventricle) mRNA expression had recovered by the first day of rearing. Combined with the fact that incubation is a period during which hens very rarely leave the nest and their social interactions may thus be very diminished, a possible role for mesotocin in the BnSTl in social interaction could explain its decrease during this reproductive stage. Unfortunately, social interaction was not measured during the experiment and this hypothesis is a possible focus for future work. Even so, it is consistent with the recovery of mRNA expression in the neuronal population under the lateral ventricle after hatch since hens would have begun interacting with their chicks at that time.

A decrease in vasotocin mRNA expression was also seen in the BnSTl with the onset of incubation but, in contrast to mesotocin, no recovery was observed in any part of the nucleus on the first day of rearing. These results can be explained by a possible attenuated stress response in the hen in both incubation and early rearing. Both vasotocin and the BnSTl have been implicated in the regulation of the stress response in birds, including the chicken (Cecchi et al., 2002; Nagarajan et al., 2014; Selvam et al., 2013), and previous studies have shown that in some animals the stress system is downregulated during the parental stage (Calisi et al., 2008; Douglas et al.,

2007; Krause et al., 2016). However, as with social behaviour, no measurement of the stress response was included in this study so this hypothesis should be tested in future studies.
The results from this project support the involvement of mesotocin in chick-rearing and suggest for the first time that vasotocin and the BnSTl may play roles in the brain of the domestic hen during incubation and rearing.

8.4 Changes in gonadotropin inhibitory hormone mRNA expression and immunoreactivity in the brain of the domestic hen throughout the reproductive cycle

GnIH and its mammalian orthologue RFRP have an inhibitory effect on reproductive behaviours and their levels are elevated when reproduction is suppressed (Amorin and Calisi, 2015; Bentley et al., 2003; Calisi et al., 2008; Ciccone et al., 2004a; Dixit et al., 2017; Li et al., 2012; McGuire et al., 2011; Moussavi et al., 2013; Small et al., 2008; Smith et al., 2008; Zhao et al., 2014), including during incubation in the chicken (Ciccone et al., 2004a; Ciccone et al., 2004b). Thus, the increase in the number of GnIH-immunoreactive neurones in the PVN of hens on the 14th day of incubation compared to egg-laying which was observed in this study was expected. More surprising was the fact that no difference was seen between any of the reproductive stages in GnIH mRNA expression in this area. This could be explained by GnIH translation proceeding at a higher rate than transcription under these conditions. It is also possible that the IHC procedure had higher sensitivity than the non-radioactive ISH used in this project.
In agreement with the hypothesis that GnIH may be responsible for inhibiting sexual behaviour during incubation, GnIH immunoreactivity and plasma testosterone changed in opposite directions during this reproductive stage. However, while testosterone concentrations decreased with the onset of

incubation (they were detectable in layers but undetectable in most incubating and rearing birds), the increase in the number of GnIH-immunoreactive neurones was not significant at that point. Therefore, further studies are necessary to determine whether GnIH is responsible for the decrease in testosterone.

In a previous study, GnIH measured throughout the reproductive cycle in the European starling was highest on the first day of incubation and the first day after hatch (Calisi et al., 2016). This differed from the pattern observed in the experiments presented in this thesis, suggesting that this hormone may act in different ways in different avian species. One possible explanation for these differences is the precocial nature of chickens in contrast to the altricial starling whose young are more dependent on maternal care. GnIH may be involved in a mechanism for the regulation of chick-rearing specific to altricial species. GnIH has been shown to be an orexigenic factor in chicks, possibly acting through the LHy (Tachibana et al., 2005). However, no evidence for this was provided by the results presented in this thesis. Food intake was not directly measured in this project but, in addition to the changes in body weight observed, there is evidence in the literature that incubating chickens have very low food intake (Hogan, 1989). Incubating hens on the 14th day of incubation and rearing hens in this study had body weights which were significantly lower compared to laying hens, while GnIH immunoreactivity in the PVN was higher during incubation. Therefore, it is unlikely that GnIH could have had an orexigenic effect in this brain area under these conditions. Contrary to expectations, no difference in GnIH mRNA expression was observed in the LHy. Another study in 56-day-old chicks also showed that fasting can elevate GnIH (McConn et al., 2016) so the possibility cannot be excluded that lower food intake could have contributed to the increased number of GnIH-immunoreactive neurones observed in the PVN. GnIH's mammalian orthologue RFRP was found to stimulate the activity of anorexigenic POMC cells in the sheep (Clarke et al., 2012) suggesting that

despite its orexigenic action in young birds GnIH may have an anorexigenic effect in adult hens in the context of incubation. In future studies, a quantification of the amount of food consumed by each bird should be included to support any conclusions about the effects of GnIH on feeding under these conditions.

8.5 mRNA expression of prolactin and dopamine receptor D2 in the pituitary gland and concentrations of monoamine neurotransmitters in the raphe nucleus of the domestic hen throughout the reproductive cycle

8.5.1 Prolactin and dopamine receptor D2 in the pituitary gland

There were no differences in the expression of prolactin or the dopamine receptor D2 in the pituitary gland of the domestic hen between any of the four reproductive stages examined, even though plasma prolactin is known to increase in birds during incubation (Cherms et al., 1962; Hall, 1991). This could be explained by prolactin being quickly released from cells. A previous study in turkeys also found no significant difference in the expression of D2 in the pituitary between laying and incubating turkey hens (Chaiseha et al., 2003) but rearing hens were not included in that study. In the study described in this thesis, D2 mRNA expression did not appear to change significantly on the first day of rearing either and may not be responsible for the decrease in prolactin after hatch observed in some avian studies (Zadworny et al., 1988).

8.5.2 Monoamines in the raphe nucleus

Some studies in mammals suggest that monoamine neurotransmitters - in particular noradrenaline, dopamine, and 5-HT - may be involved in maternal behaviour (Byrnes et al., 2002; Calamandrei et al., 1992; Cox et al., 2011; Rosenberg et al., 1977; Rothlin et al., 1922; Steele et al., 1979; Tanaeva et al., 2012; Harding and Lonstein, 2016). However, no differences were observed in the concentration of any of these compounds or their precursors in the raphe nucleus where they are known to be synthesised (Byrnes et al., 2002; Saavedra et al., 1976; Tanaeva et al., 2012; Versteeg et al., 1976; Harding and Lonstein, 2016). Previous data suggest that noradrenaline is important mostly for the onset of maternal care, while dopamine (Byrnes et al., 2002; Tanaeva et al., 2012; Alston-Mills et al., 1999) and 5-HT (Harding and Lonstein, 2016) may play a role further in rearing. It has been suggested previously that the dopaminergic system may be important for interactions between the mother and the young (Curry et al., 2013) so it is possible that a difference in dopamine and 5-HT concentrations would have been present if the hens included in this project had been allowed to rear their chicks for a longer period of time.

Unfortunately, husbandry issues made it impossible to procure more tissue for experiments and led to suboptimal conditions when processing the available tissue. This may have interfered with the sensitivity of the LC-MS assay and led to the high intra- and inter-assay variability observed. Therefore, these results should be interpreted with caution and the possibility that monoamines in the raphe nucleus are involved in the control of maternal behaviour in the domestic hen should not be excluded.

8.6 The effects of interactions with chicks on the behaviour and neuronal activation the brain of the adult female Japanese quail

The experimental setup used in this project - 6-day-old chicks roaming freely in the pen as opposed to day-old chicks placed under hens during the night in a confined space, which was an approach used in previous studies (Richard-Yris, 1983; Ruscio, 2004; Richard-Yris, 1987) - did not induce full maternal behaviour in adult female Japanese quail. However, after habituation to chicks for 6 days, experimental birds spent significantly more time in proximity to novel chicks than to a novel adult. It is possible that this favourable social response restricted to young conspecifics may preclude maternal behaviour. A significant difference was indeed found in the number of nuclei immunoreactive for c-fos in the PVN between the two experimental groups, but double-labelling showed that the majority of these nuclei were not colocalised with mesotocin neurones. Therefore, the cells contributing to this difference remain unknown. One possibility is that GnIH neurones which, as already established, are found in the PVN and play a role in the inhibition of reproduction (Bentley, 2006; Johnson, 2007) become activated by interactions with chicks in order to decrease sexual behaviour in preparation for maternal behaviour.

Another area where Fos-immunoreactive nuclei were more numerous in quail presented with novel chicks than quail presented with a novel adult was the nCPa. This area has been shown to have an abundance of GnRH neurones and be involved in the control of reproductive behaviours (Fraley and Kuenzel, 1993; Kang et al., 2006; Thayananuphat, 2007; Sartsoongnoen, 2012; Chaiyachet, 2013a). These results are surprising as GnRH neurones were not expected to become activated in the presence of chicks. GnIH neurones were also found in the nCPa in the chicken in one study (Zhang et al., 2017), but they were low in number. As suggested above, it is possible

that GnIH neurones in this area act to inhibit sexual behaviours when chicks are present. However, since no sections were left to double-label cells in the nCPa for c-fos and GnRH or c-fos and GnIH, it remains to be discovered what type of neurones are responsible for the observed difference between groups. In contrast to the results in the PVN and nCPa, a lower number of Fos-imunnoreactive nuclei were found in the raphe nucleus of quail interacting with novel chicks compared to quail interacting with a novel adult. 5-HT neurones in the raphe nucleus have been previously shown to promote the care for younger siblings in virgin rats (Harding and Lonstein, 2016) so a decrease in neuronal activity in this area in the presence of young was unexpected. However, 5-HT has also been shown to increase anxiety (García-García et al., 2017; Pereira et al., 2017). It is possible that for adult quail females which have previously interacted with both chicks and other adults, chicks present a less stressful stimulus, causing less stress-related activation in the raphe nucleus.

8.7 Criticism of experiments, obstacles and future work

8.7.1 The effect of husbandry issues on the experiments presented in this thesis

While it is the belief of the author that this thesis presents valuable novel data on the control of maternal care in avian species, it should be noted that many of the aspects of this PhD project were greatly negatively affected by circumstances beyond the author's control. The experiments depended on the availability of a strain of bantam chicken - the Silkie - which displayed maternal behaviour in order to create the F1 Silkie and White Leghorn cross which was required. To the author's knowledge, these birds were readily

available from the Roslin Institute Poultry as a colony and had been maintained there for years. Unfortunately, after the successful completion of the first planned experiment on maternal birds, when attempts were made to hatch the necessary number of hens for the next experiment, it became apparent that the parent stock were no longer fertile. Numerous time-consuming attempts were made to overcome this issue. These included allowing the male Silkies to 'rest' while kept on short days in the hopes of increasing sperm production and then once again attempting artificial insemination, observing another strain of chickens for displays of maternal behaviour, and most importantly, trying to procure new birds to regenerate the colony. This proved very difficult for several reasons. Firstly, as Silkies are not commercial birds and are mostly kept as backyard chickens, they are not widely available. Once a reliable supplier was identified, safety concerns had to be overcome. Veterinary regulations at the Roslin Institute imposed restrictions on the import of such birds into the avian facility, as their health status could not be easily confirmed. A method was agreed on for the procurement and hatching of new Silkies which involved testing the parent stock at their home farm for common pathogens, treating any infected birds before procuring fertile eggs, and then placing the eggs and hatchlings under quarantine until they, too, could be tested. However, this was further delayed by the fact that the resident colony of Japanese quail had been lost in a similar way prior to the Silkie colony and the new quail were undergoing similar procedures. Thus, before that colony could be regenerated, the quarantine facilities were not available. This also explains why experiments on quail could not be started sooner and not enough time and birds were available to develop a better experimental setup. Attempts to procure new Silkie chickens ultimately failed due to issues at the supplying farm which, after all previous delays, delayed acquiring the birds beyond the point where it would have been possible to hatch and use them in this project. This necessitated the use of tissue from the single experiment in maternal

chickens which was successfully completed for a variety of purposes. It became impossible to conduct some of the experiments which had been originally planned so the scope of the project was broadened to include a wider variety of compounds. Thus, while it is acknowledged that some of the experiments in this thesis suffer from the obstacles encountered, the author would like to make it clear that every effort was made to overcome these obstacles and obtain valuable data.

8.7.2 Improvements to present experiments and future work

There are certain changes that can be made to the experiments presented here in order to improve the quality of the data. In addition to that, these experiments present a basis for future work.

The ISH revealed mRNA expression for mesotocin and vasotocin in the hyperstriatum ventrale (HV) of the domestic hen - a region which has been shown to play a role in imprinting in chicks (Horn et al., 1979). As nonapeptides are known to play a role in learning and memory (Bartus et al., 1982; Bohus et al., 1978; Davis and Pico, 1984; Gibbs and Ng, 1984), it is possible that mesotocin and vasotocin in this brain area may be involved in this process. Further studies on the HV of chicks during imprinting, measuring nonapeptide expression under natural conditions, as well as observing the effects on imprinting of nonapeptide antagonists, knockout or gene silencing would help test this hypothesis. Additional brain areas where the effects of nonapeptides should be further studied in birds include the hippocampus (Hp) and the olfactory tubercule (Tu). In these areas, receptor-binding autoradiography revealed strong bidning signal for the mesotocin (VT3) receptor. The Hp plays a role in spatial cognition (Morris et al., 1982; Balda and Kamil, 1992; Biegler et al., 2001). Therefore, it is not unreasonable

to propose that mesotocin in birds may have an effect on memory through its receptors in this area, as mesotocin's orthologue oxytocin is involved in memory in mammals (Bohus et al., 1978; Kovács et al., 1979; Lukas et al., 2013). In the case of the olfactory turbercule, mesotocin in this area may be involved in the control of social behaviour. The Tu has a role in processing sensory and social stimuli (Hitt et al., 1973), as well as social behaviour in the rat (Koob et al., 1978) in addition to being connected to the reward centers in the brain (Ikemoto et al., 2003).

One of the areas examined which has been neglected in previous studies was the BnSTl. Measuring the mRNA expression and immunoreactivity of mesotocin and vasotocin neurones in the BnSTl in stress and in different social contexts could help us better understand possible roles of nonapeptides in this nucleus in both mammals and birds. Examining the effect of lesions in this brain area on stress and sociality could also help determine its importance. Future studies should be conducted in both juveniles and adults as findings in young animals which have not reached sexual maturity do not necessarily apply to older individuals. However, as the BnSTl had not been examined before, data on the effects on juveniles is of value regardless. In addition, for the purpose of this project, juvenile birds were a cheaper alternative to gonadectomised adults and the results for the effects of sex steroids on vasotocin described int his thesis corresponded well with result obtained from adult gonadectomised quail. This suggests that, in the context of the effect of sex steroids, juveniles may be an adequate replacement. Comparing these data with data from future studies on adults will show whether the response of the mesotocin and vasotocin systems in this brain area changes with age.

Studies on the response of BnSTl nonapeptide neurones to stress can be combined with the administration of sex steroids to determine their effects under these conditions. In the experiments presented in this thesis, the

effects of sex steroids on behaviour were not measured. Behavioural studies including measuring stress and aggression together with changes in the mesotocin and vasotocin system after steroid treatment should be included in future work. The mechanisms through which sex steroids affect nonapeptides also require further study. To test the hypothesis that testosterone acts on brain nonapeptides through estradiol after aromatisation, experiments involving the delivery of aromatase inhibitors to the brain can be conducted. A detailed characterisation of gonadal steroid receptors in the brain of the chicken would be very useful, as well as Double-labelling for these receptors and mesotocin or vasotocin in order to find out whether they are present on nonapeptide neurones themselves or their afferents. This would help determine the mechanism of action of sex steroids on nonapeptidergic systems. Mesotocin receptor binding in the brain after steroid administration should also be examined, not only in juveniles but also in adult birds in order to establish whether there is a difference. Unfortunately, vasotocin receptor binding in the chicken brain could not be quantified as attempts were unsuccessful to find a ligand that worked for this receptor in the chicken. Hopefully, in the future it will be possible to obtain a suitable ligand and develop an autoradiographic procedure that would allow for the quantification of changes in binding to receptors more specific to vasotocin.

While this project focused on the PVN and BnSTl, other brain areas containing mesotocinergic and vasotocinergic neurones should also be examined for effects of sex steroids on those neurones. The changes in mRNA expression throughout the hypothalamus for both mesotocin and vasotocin at different time points in the domestic hen's reproductive cycle were characterised in this thesis. However, while the IHC procedure used for mesotocin immunoreactivity in the PVN produced quantifiable results, the thinness of the sections used did present an issue, as some damage to the tissue was observed and sometimes necessitated for samples to be discarded. It was also impossible to visualise mesotocin fibers. An attempt

was also made to obtain data on the vasotocin immunoreactivity in the PVN but none of the antibodies tested produced satisfactory results, as staining was too faint to quantify. Using thicker fresh frozen or fixed sections in the future would improve the sensitivity of the procedure for mesotocin and hopefully allow for the quantification of vasotocin immunoreactivity.
Since the number of sections available was limited, immunoreactivity could not be examined in tandem with mRNA expression for mesotocin and vasotocin throughout the reproductive cycle in hens so this should be addressed in future work. It may also be beneficial to widen the time range of experiments to include a time point later in rearing than the first day after hatch.

The importance of nonapeptides for incubation and chick-rearing in the chicken can be better understood by blocking their effects with the help of antagonists or using techniques such as gene knockout and gene silencing. Indeed, combining various methods for blocking the effects of a peptide together with behavioural observations should be applied to all compounds investigated in this PhD project. For example, examining the effects of blocking GnIH in the brains of incubating hens or hens rearing chicks would help determine whether this peptide has any importance in incubation or in rearing behaviour in the chicken. In the case of feeding, as the orexigenic effects of GnIH have only been studied in chicks, further experiments are needed to determine its effects in adult birds, including characterising c-fos expression from GnIH neurons in both the PVN and LHy in mature chickens at different reproductive stages.

Attempts made during this project to measure the mRNA expression for the D1 dopamine receptor in the pituitary gland were unsuccessful. This was due to the fact that expression was below the detection limit of the qPCR procedure and tissue from the hypothalamus where this receptor is more highly expressed was not available. In future studies, the mRNA expression

and immunoreactivity for this receptor in the hypothalamus should be measured throughout the hen's reproductive cycle together with prolactin and D2 mRNA expression and immunoreactivity and prolactin plasma concentrations.

As the tissue used for measuring monoamines in the raphe nucleus was not prepared in the optimal way, improvements in the protocol in future studies should include more precise dissection of the raphe nucleus and the absence of repeated freezing and thawing. This may improve the accuracy of the assay. Once again, agonists and antagonists, as well as gene knockout and gene silencing can be used in combination with behavioural studies to better understand the importance of monoamines in maternal behaviour.

As described above, husbandry and logistic issues, as well as animal welfare considerations had an impact on the study design for the effects of chicks on the behaviour of adult female precocial birds and the activation of neuronal systems in the brain with maternal care. This experiment was originally developed for chickens. However, due to the loss of the only strain of chickens at the Roslin Institute Poultry Unit which displayed maternal behaviour and the failure of any attempts to recover it, the experiment was redesigned for quail as the only other available birds capable of becoming maternal. The fact that Japanese quail do not incubate in captivity presented a problem, as in the original study design young chicks would have been introduced to hens at different time points during incubation, including the predicted day of hatch. Chickens have been shown to display full maternal behaviour towards foster chicks as early as day 10 of incubation (Richard-Yris et al., 1987) but in the absence of incubation, inducing the behaviour in quail was more difficult. The use of previously used paradigms was also not possible in this project due to constraints on time, equipment, experimental rooms and numbers of birds, as well as ethical considerations for the safety of young chicks. While it is the belief of the author that the results presented

here are valuable, given more time and the appropriate birds, improvements can be made. In addition to completing the originally planned experiments with chickens, the study in quail presented here should be expanded to include periods of longer cohabitation with chicks of various ages and the induction of full maternal behaviour. Aside from improving the experimental setup, the main goal of future work should be to identify the cells responsible for the differences in immunoreactive c-fos observed in the PVN, nCPa and the raphe nucleus with the help of double-labelling IHC for c-fos and neuropeptides known to be expressed in these areas. In order to find out if the lower neuronal activity in the raphe nucleus in the presence of chicks as opposed to a novel adult suggested by the results presented here is connected to how much of a stressor each of these stimuli is, measurements of plasma corticosterone and data on stress-related behaviours should be obtained under the same conditions.

Conclusion

Maternal behaviour in birds is tightly connected to reproduction, feeding and the stress response and is controlled in the brain by complex interactions between a number of neuropeptides involved in social and reproductive behaviours. New brain areas of interest which may be involved in the control of maternal care have emerged from this study, one of which is the BnSTl. While the BnSTm is known to be involved in social and maternal behaviour, few studies have focussed on the BnSTl and its role in this behaviour remains unclear. However, the findings from this project present an opportunity for further investigation of the BnSTl in relation to the maternal stress response in avian species, as well as other functions during incubation. In addition to the BnSTl, the data from this project present evidence that neurons in the nCPa, which is usually implicated in the control

of reproduction, become activated in the presence of chicks. However, the identity of these neurones and their involvement in interactions with chicks remains to be discovered.

Furthermore, these results broaden our understanding of the actions of several neuropeptides. Nonapeptides are well-established regulators of social behaviours, including maternal behaviour in mammals, but little is known about their importance in avian maternal care. The findings presented in this thesis support the hypothesis that mesotocin in the PVN is important for chick-rearing and present new evidence that the mesotocin and vasotocin systems in the BnSTl may be indirectly involved in incubation as nonapeptide mRNA in this area decreased during this reproductive stage in the domestic hen. Results also show that both mesotocin and vasotocin can be elevated by gonadal steroid hormones in areas of the brain behaviour network in the chicken and provide the first data on the effects of gonadal steroids in the BnSTl.

The link between reproduction and maternal behaviour appears to be under the control of GnIH in some species but data on the role of this peptide in maternal care are limited, especially in precocial bird species. The data from this project suggest that a difference may exist in the control of chick-rearing between precocial and altricial birds and GnIH may be involved in this difference.

In conclusion, the significance of this project lies in furthering our understanding of known neuronal systems involved in the control of maternal care as well as presenting evidence for the involvement of less studied brain areas and neuropeptides. Future studies of these systems and peptides will provide valuable data contributing to our understanding of the neuroendocrine control of maternal care in birds.

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